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Optimization of the production of motoho, a fermented sorghum beverage

Sanchia S. Moodley

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

In the Faculty of Science

Department of Microbiology and Cell Biology

University of the Witwatersrand, Johannesburg

Supervisor: Prof Vincent Gray (University of the Witwatersrand)

Co- Supervisor: Dr Lucia Steenkamp (CSIR, Biosciences)

Date: 29 May 2019

Declaration

I, Sanchia S. Moodley with student number 9700783N, am a student registered for the degree of Doctor of Philosophy in the academic year 2018.

I hereby declare the following:

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- I confirm that the work submitted for assessment for the above degree is my own unaided work except where explicitly indicated otherwise and acknowledged.
- I have not submitted this work before for any other degree or examination at this or any other University.
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Sanchia S. Moodley



Date: 29 May 2019

Summary

Traditionally fermented products are developed by local inhabitants of a region and the production method is handed down through generations. Motoho is one such product which is a traditionally produced fermented sorghum beverage indigenous to the Basotho people of Southern Africa. The aim of this research was to ferment and cook motoho using four different sorghum varieties in the presence of either a traditional or a defined starter culture which comprised *Lactobacillus fermentum* and *Lb. plantarum*, and to evaluate the influence of motoho fermentation on the tannin and phytate contents of sorghum. Sixteen motoho variations were screened using Inductively Coupled Plasma Sectorfield Mass Spectrometer for iron (Fe), zinc (Zn), phosphorous (P), calcium (Ca) and magnesium (Mg) content. The traditional motoho (A) produced the highest Fe (45.91mg/Kg-^{1}) and Ca contents (385.57mg/Kg-^{1}) while the motoho produced with Rakodzi sorghum (J) showed the highest Zn concentration (26.62mg/Kg-^{1}). The motoho produced with SC Smile sorghum (M) yielded the highest P and Mg contents of 3894.96 and 1768.20 mg/Kg-^{1} respectively. Only these motoho varieties (A, J and M) were selected for further analysis.

Motoho A was made using regular commercial sorghum and the traditional starter culture called Tomoso, while J and M were made using Rakodzi and SC Smile sorghums respectively as well as the defined starter culture. Compared to the raw sorghums, the tannin contents of the selected motoho were reduced by up to 94%. However, even though endogenous phytase was measured during motoho production the values were low (0.26-0.44U/ml) and the phytate contents were reduced by up to 9.3 %. The mineral bioavailability of Fe, Zn, Ca and Mg of the selected motoho varieties were conducted using Caco-2 cell uptake assays. The Fe (0.29-0.51%), Zn (0.43-0.44%), Mg (0.87-1.28%) and Ca (10.47-26.58%) uptake percentages could not meet the recommended daily intake for infants up to 12 months.

Different pre-processing methods for example decortication, soaking and germination have been shown to activate endogenous phytates and could be used to decrease the

phytate contents of motoho thereby increasing the mineral bioavailability since phytate has been shown to inhibit Fe and Zn bioavailability.

The current research also aimed to determine the effects of using the defined starter culture comprising of *Lb.* and *Lb. plantarum* compared to the traditional production, on the sensory qualities and shelf-life of motoho. Motoho varieties A, J, and M were subjected to shelf-life studies as well as sensory analysis. The volatile organic compounds produced during fermentation were also analyzed. Samples were subjected to 4ºC and 37ºC storage for the shelf-life study while a sensory panel assessed the acceptability of each motoho variety. The microbiological counts for A, J and M incubated at 4 ºC were 1 log cfu/ml, 1.48 log cfu/ml and 1.3 log cfu/ml on day 5 respectively. The counts for A, J and M incubated at 37ºC were 3.48 log cfu/ml, 3.18 log cfu/ml and 3.01 log cfu/ml on day 5 respectively. The largest amounts of ethyl and methyl esters were produced during the fermentation of J which could account for the preference in aroma and flavour by sensory panellists. Aldehydes were found in the fermentation of A while acetoin and butanediol were produced during the fermentation of M. Panellists also preferred the appearance of J but the mouth feel of M.

The motoho variety J appears was the most preferred in terms of flavour and aroma. It also had the highest Zn concentration as well as having the highest uptake percentage for Mg, Ca and Fe during the Caco-2 uptake assay. Future research on motoho could include the motoho variety J and could include methods to decrease the phytate content and thereby improve the mineral bioavailability of motoho. This research has also shown that along the selected lactic acid bacteria (LAB) strains which were used for this research could be used for further research on motoho in order to upscale the production and possibly commercialize the product as it has shown, in particular the motoho variety J, to be a highly favourable product with an extended shelf-life.

Dedication

For Raeés, Yvonne and Peter

Psalms 23

The Lord is my Shepherd, I shall not want
He makes me to lie down in green pastures
He leads me beside the still waters
He restores my soul
He leads me in the paths of righteousness
For His name's sake

Yea though I walk through the valley of the shadow of death
I will fear no evil
For you are with me
Your rod and Your staff, they comfort me

You prepare a table before me in the presence of my enemies
Mu cup runs over
Surely goodness and mercy shall follow me all the days of my life
And I will dwell in the house of the Lord
Forever

Research Outputs

Original Publications

Publications which form part of the thesis:

Title: Sensory analysis and organic flavour compound production during the fermentation of motoho, a sorghum beverage

*Sanchia S. Moodley[1], Lucia Steenkamp[1] and Vincent M. Gray[2]

[1]CSIR Biosciences, PO Box 395, Pretoria, Gauteng 0001, South Africa

[2] Department of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg, 2050, South Africa

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Conference Presentations

1st International Conference Sorghum in the 21st Century April 2018, Cape Town, South Africa Poster Presentation

Title: Optimization of the production of motoho, a fermented sorghum beverage

Authors: Sanchia Moodley, Lucia Steenkamp, Vincent Gray

4th BISMis International Conference

April 2018, Muldersdrift, South Africa

Poster Presentation

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Authors: Sanchia Moodley, Lucia Steenkamp, Vincent Gray

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To my family and friends, I thank you for your love and constant support.

To Raeés, thank you for being my strength throughout this journey.

To my parents, you are always loved and remembered.

Thank you to my Lord and Saviour for Your grace, mercy and favour.

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List of abbreviations

˚C	degrees Celsius
(-)	negative
%	percentage
<	less than
≤	less than or equal to
≥	greater than or equal to
ATCC	American Type Culture Collection
Ca	Calcium
cm^2	centimetres squared
CO_2	carbon dioxide
cfu/ml	colony forming units per millilitre
CSIR	Council for Scientific and Industrial Research
E.	*Escherichia*
FAO	Food and Agriculture Organization
Fe	Iron
GIT	gastrointestinal tract
g	gram
g Kg-1	gram per kilogram dry weight

g/100g	gram per one hundred gram dry weight
GC-MS	Gas Chromatography Mass Spectrophotometer
hr	hours
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
ICP-SFMS	Inductively Coupled Plasma Sectorfield Mass Spectrometer
LAB	Lactic acid bacteria
Lb	*Lactobacillus*
Lc	*Lactococcus*
LTB	Lauryl Tryptose Broth
m	meter
mM	millimolar
M	molar
Mg	Magnesium
mg	milligram
mg Kg-1	milligram per kilogram dry weight
mg/100g	milligram per one hundred gram dry weight
mg/ml	milligram per millilitre
mg/100ml	milligram per 100 millilitre
min	minute
ml	millilitre
ml/min	millilitre per minute
mm	millimetre
mol	moles

m/z	mass-to-charge ratio
MPN	Most Probable Number
MRS	de Manne, Rogosa and Sharpe agar
MRD	Multiple Recovery Diluent
NaCl	sodium chloride
NaOH	sodium hydroxide
NaHCO3	Sodium bicarbonate
O_2	oxygen
PDA	Potato Dextrose Agar
rcf	relative centrifugal force
rpm	revolutions per minute
sac.	*Saccharomyces*
sec	seconds
SME	Small and medium sized enterprises
sp.	species
TEER	Transepithelial/endothelial electrical resistance
TPC	Total Plate Count
TSA	Tryptone Soy Agar
U	units
U/ml	units per millilitre
u/gds	units per gram dry substrate
µg	microgram
W.	*Weisella*
WHO	World Health Organization

µg/ml	microgram per millilitre
µl	microliter
µM	micromolar
µm	micrometre
uv	ultraviolet
v	volts
vol/vol	volume per volume
w/v	weight per volume
P	Phosphorous
Zn	Zinc

1 Introduction

Fermented products are sought-after, and are considered to be safe for consumption by rural dwellers (Food and Agriculture Organization, 2011) allowing the small-scale fermentation of products to be a means of creating an income (FAO, 2011). Motoho is a spontaneously fermented non-alcoholic sour porridge or beverage produced by the Basotho (people of Lesotho). Spontaneous fermentation is an uncontrolled process which depends upon microorganisms originating from the raw material, utensils, or added as a starter culture (Nout and Motarjemi, 1997) which often results in fermentation failure, unpredictable microbiological flora, or delayed fermentations. In order to upscale the production of fermented foods from a household to a more industrialized level and to produce end-products with consistent quality , the microorganisms involved in the fermentation need to be isolated and categorized and their function in the fermentation needs to be well-defined (FAO, 2011).

Motoho is produced with sorghum which has poor nutritional quality and consists of anti-nutrients like phytates, phenolic compounds and tannins. (Léder, 2004) making it undesirable in targeting malnutrition, a global health issue which can cause morbidity, impaired intellectual development and working ability as well as mortality (Michaelsen et al., 2009).

The thesis is divided into four chapters. The first chapter, the literature review aims to highlight the ways to combat the inconsistency in the production of motoho by exploring the use of starter cultures. The anti-nutritional content of sorghum and also ways to reduce it are discussed. This is important for the optimization of motoho which may contribute to its commercialization and thereby aid micronutrient malnutrition. The remaining two chapters are the research chapters and the last chapter concludes the thesis. The referencing style for the research chapters follow the American Psychological Association (APA), which is in line with the requirements for the selected journal while the remainder of the thesis follows the Harvard referencing format.

2 Literature Review

2.1 Malnutrition

Child malnutrition is a global health issue which can cause morbidity, impaired intellectual development and working ability as well as mortality (Michaelsen et al., 2009). Malnutrition can be attributed to inadequate amounts of food intake and poor nutritional quality of the food which is available (Brown, 1991) especially amongst plant-based diets which contains low amounts of micronutrient rich animal food sources (Hotz and Gibson, 2007). Malnutrition includes over and undernutrition. Undernutrition is defined as micronutrient deficiency or protein energy malnutrition while over nutrition is ingesting an excess of macronutrients or energy (Faber and Wenhold, 2007). Micronutrients are constituents of foods required in small quantities and are important for human health (Miller and Welch, 2013). They comprise vitamins and important trace elements. When the bioavailable micronutrient intake cannot meet requirements, micronutrient malnutrition develops with iron (Fe), vitamin A and iodine deficiency being the most prevalent (Allen et al., 2006). Vitamin B12 and zinc (Zn) deficiency is also widespread (Miller and Welch, 2013). Micronutrients have a role in reproductive health, humoral and cellular immune responses, learning and cognitive functions, cellular functions and signalling (Guerrant et al., 2000; Kapil and Bhavna, 2002). These micronutrients cannot be created by the body and must be supplied by means of the diet (Kapil and Bhavna, 2002).

Food diversity, under ideal conditions of food accessibility and availability, should be able to provide the energy and micronutrient needs of the general population (FAO, 1997). However, many people worldwide do not have access to micronutrient-rich foods. Foods with a high micronutrient density such as green leafy vegetables, pulses, legumes and fruits, will ensure proper nutrition, including adequate micronutrients, for most populations (FAO, 1997).

Populations with a high prevalence of malnutrition, characteristically have diets which comprise mainly starch like cereal (maize, rice) or tubers like cassava, are bulky and have low nutrient and energy density as well as low mineral bioavailability (Michaelsen et al.,

2009). Worldwide, approximately 45% of child deaths are the result of malnutrition (Black et al., 2013). Micronutrient deficiency and sub-optimal breast feeding results in over ⅓ of these deaths, and is attributable to 11% of the disease setback globally (Bhutta et al., 2013).

2.2 Food Fortification

Food fortification and food supplementation are substitutes which supplement food-based methods of satisfying the nutritional needs of the populations in both developing and developed countries (FAO, 1997).

Fortification is the addition of nutrients to a food (the vehicle) which is commonly eaten. One or many nutrients (the fortificant) can be added to the vehicle. The food selected as a vehicle for fortification must be inexpensive, widely distributable to target communities countrywide, should contain sufficient nutrient levels after processing and must be consumed by large amounts of the population, especially lower income groups. This food should also not have an unpleasant taste, colour or appearance after fortification (FAO, 1997).

2.3 Iron

Iron has many important functions in the body. It carries oxygen (O_2) to tissues from the lungs by way of red blood cell haemoglobin, is a transporter of electrons within cells and is a fundamental component of enzyme systems in different tissues (Gupta, 2014). Infants require very high Fe levels in relation to energy requirements during the weaning period. Iron nutrition is important for brain and tissue development. Iron deficiency is an absence of Fe stores accompanied by signs of Fe deficiency erythropoiesis which means that there is an inadequate supply of Fe to certain tissues (Joint FAO, 2002). Iron deficiency is the most recurrent nutritional deficiency and arises when the diet cannot provide the physiological Fe requirements with around 600-700 million people worldwide having Fe deficiency anaemia (Densaeyer and Adiels-Tegman, 1985), with a high prevalence in developing countries. The highest incidence of Fe deficiency worldwide is in infants, women of child-bearing age, children and adolescents (Joint FAO, 2002).

Iron is present as haem Fe which can be derived from flesh foods such as meat, fish and poultry and is found in the reduced state (Fe^{2+}), allowing for greater absorption than oxidised non-haem Fe (Fe^{3+}) which is mostly found in meat and plant based foods (Maret, 2000). The main dietary Fe form is non haem Fe. Haem Fe absorption varies from 40% when Fe is deficient to 10% when Fe is replete (Hallberg et al., 1997). Calcium (Ca) can negatively influence the absorption of both haem and non-haem Fe (Hallberg et al., 1993). Several dietary factors as well as an individual's Fe status can affect non haem Fe absorption (Joint FAO, 2002). Reducing substances which maintain Fe in the ferrous form must be prevalent for Fe absorption (Wollenberg and Rummel, 1987) while dietary poultry, meat and fish enhance Fe absorption. Other foods contain ligands such as Fe-binding polyphenols and phytates which prevent Fe absorption by strongly binding ferrous ions (Joint FAO, 2002).

2.4 Zinc

Zinc is found in all body tissues and fluids and is an essential part of many enzymes which are involved in the synthesis and degradation of proteins, nucleic acids, lipids, carbohydrates and also in the metabolism of micronutrients (Joint FAO, 2002). The molecular structure of membranes and cellular components are stabilized by Zn and in this way, Zn maintains cell and organ integrity. Zinc also has a role in polynucleotide transcription and hence genetic expression (Joint FAO, 2002). Zinc deficiency can result in growth retardation, alopecia and a decreased immunity and an increased sensitivity to infections (Copper, 2001; Shils and Shike, 2006).

2.5 Zinc fortification

Zinc stores are limited so the body depends on regular Zn supplies obtained from the daily diet. Zinc adequacy cannot be achieved in the absence of flesh foods of which beef is the best source. Zinc fortification programmes are important for populations which consume mostly plant foods. Stunting caused by low Zn diets in developing countries could be reduced by the addition of Zn to the diet through fortification of staple foods (Joint FAO, 2002).

2.6 Calcium

The human skeleton comprises about 99% of the body's Ca, with the remaining 1% being distributed between soft tissues and teeth. Ionized Ca (4-8mg/100ml) is found in the intracellular fluid (ECF) with protein-bound Ca in the plasma (3.2mg/100ml) (Joint FAO and World Health Organization, 2005). Calcium ions play a part in most metabolic processes while Ca salts give the skeleton rigidity.

Calcium plays an essential role in blood clotting, enzyme-mediated processes, neuromuscular functions as well as giving the skeleton rigidity through its phosphate salts (Joint FAO, 2002).

2.7 Phosphorous

Phosphorous (P) or phosphate is part of the phospholipids, a vital, functional part of cell membranes and part of high energy phosphate compounds like adenosine triphosphate and creatine phosphate which are energy saving molecules, important for all essential processes. Phosphorous is an important part of hydroxyapatite, the key structural bone material (Michaelsen et al., 2009). Phosphorous deficiency is common in malnourished children (Manary et al., 1998) and may lead to rickets-like bone changes. Fish, meats, poultry, nuts, eggs and dairy are good sources of available P. Phytate is the main form of P from plant material and is impervious to digestion unless broken down by the phytase enzyme. Under normal conditions, P from phytate is minimally absorbed (Golden, 2009) Anti-nutrients like polyphenols, phytate and oxalate also limit the bioavailability of nutrients in a primarily plant-based diet (Gibson et al., 1998; West et al., 2002).

In developing countries, various forms of malnutrition in adults and children result from inadequate protein intake. The best protein sources are animal sources which are expensive in sub-Saharan African countries so the dependence on plant protein is high (Adebayo-Oyetoro et al., 2012) However, plant proteins are lacking some of the essential amino acids (Hoffman and Falvo, 2004). A sizable fraction the cells phosphate is also found in mRNA and thus plays a key role in the turnover of proteins.

2.8 Sorghum Nutrition

Sorghum (Sorghum bicolor (L.) Moench) is a drought-tolerant cereal which requires minimal input during growth but which provides a better yield with good husbandry (International Crops Research Institute for the Semi-Arid Tropics and FAO, 1996). In terms of global production, sorghum is the 5th most important cereal after maize, wheat, rice and barley (Taylor and Emmambux, 2008). Sorghum grain (Figure 1) is spherical and roughly 4mm in length with varying colour from virtually white to virtually black, with brown and red shades quite common. In many varieties of sorghum a thick testa layer under the pericarp contains condensed tannins.

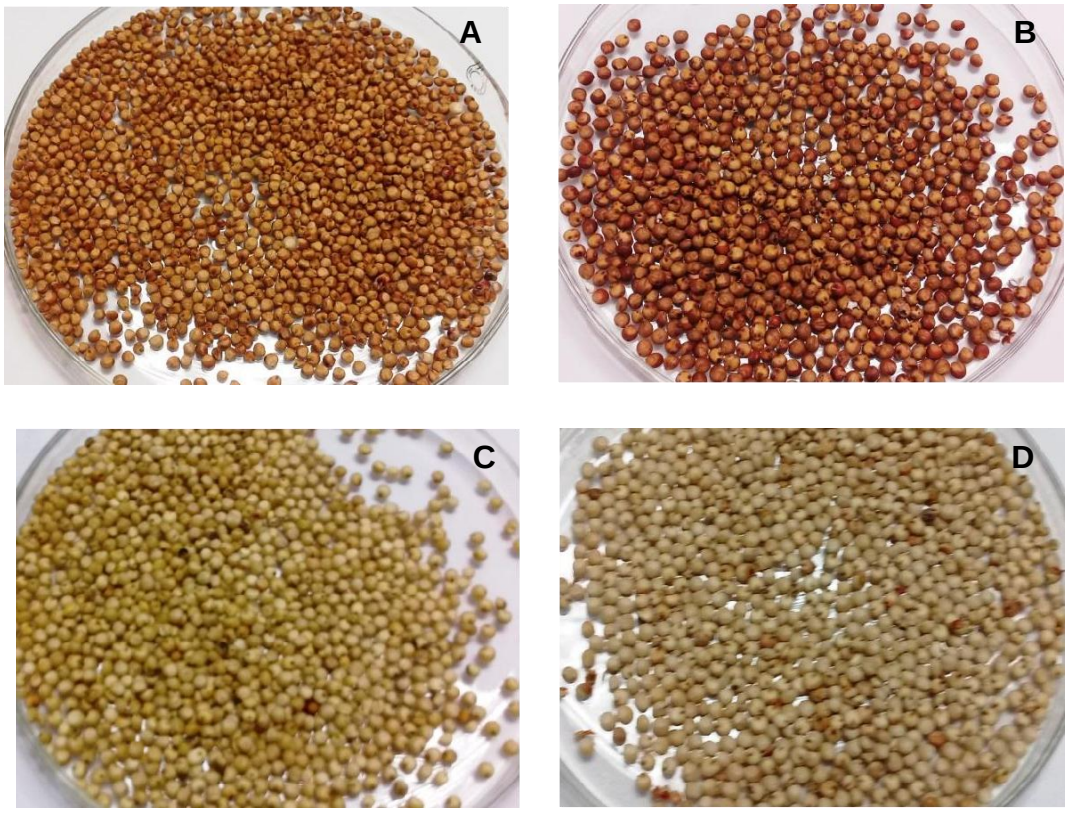


Figure 1: Sorghum varieties A (SC Smile), B (Rakodzi), C (SV Smile) and D (Macia) obtained from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) Zimbabwe and used for the fermentation of motoho during the present study. Regular commercial sorghum was used as the control. Image by S. Moodley ©, Council for Scientific and Industrial Research (CSIR), Pretoria, South Africa, 2018.

In most developed countries, sorghum is underutilized and used mainly for animal feed while it is an important crop for millions of people in parts of Asia and Africa. Sorghum has low

amino acid content (lysine, threonine, tryptophan) and hence a poor protein quality (Badi et al., 1990). After cooking, sorghum proteins become less digestible (Axtell et al., 1981; Eggum et al., 1983). Infants digest sorghum poorly (MacLean et al., 1983) but it can be used as an adequate weaning food if combined with foods high in lysine (Badi et al., 1990). Sorghum usually contains roughly 11% protein but this is extremely variable. The lysine content is approximately 2.0g/100g (Awika and Rooney, 2004). In high tannin sorghum varieties protein utilization is limited due to the formation of indigestible protein complexes (Chibber et al., 1980) while reports have shown a hindrance to Fe absorption in the gastro-intestinal lumen due to phenolic acids and flavonoids (Brune et al., 1989). Many varieties of sorghum have large amounts of phenolic compounds with strong antioxidant activity (Awika and Rooney, 2004) with condensed tannins displaying the highest *in vitro* antioxidant activity (Hagerman et al., 1998; Amarowicz et al., 2003). Potassium (K) and P are the main minerals found in sorghum, which has low Ca values (Khalil et al., 1984).

2.9 Phytates and phytases

Phytates are used to describe the hexaphosphate esters of inositol and naturally occur in a large number of roots, tubers and plant seeds (Harland and Oberleas, 1985). They form insoluble phytate-mineral complexes with minerals (Dvořáková, 1998), thereby decreasing mineral bioavailability (Kumar et al., 2010). In sub-Saharan Africa, Zn and Fe deficiency is widespread and households largely consume cereal based diets (Uusiku et al., 2010) which are high in phytates (Kruger et al., 2015). Phytate (IP6) is storage for P in seeds and is important during germination for P release. Phytates are accountable for up to 80% of the overall P content of seeds and 1.5% of dry weight (Michaelsen et al., 2009). The structure of phytate can be seen in Figure 2. It is hydrolysed by endogenous phytase during germination, releasing inositol and phosphate (Bohn et al., 2008). Phytate and other phosphorylated inositol's (IP1-5) can be found in minute quantities in the majority of mammalian cells where they participate in different functions relating to cell signalling pathways (Michell, 2008). The human digestive system is unable to break down phytates. The phosphate groups in phytate bind strongly to the divalent cations of Fe, K, Mg, manganese, Zn, and Ca. IP4 and IP5 form weaker complexes than IP6 with Fe and other metals (Lönnerdal et al., 1989; Sandberg et al., 1999).

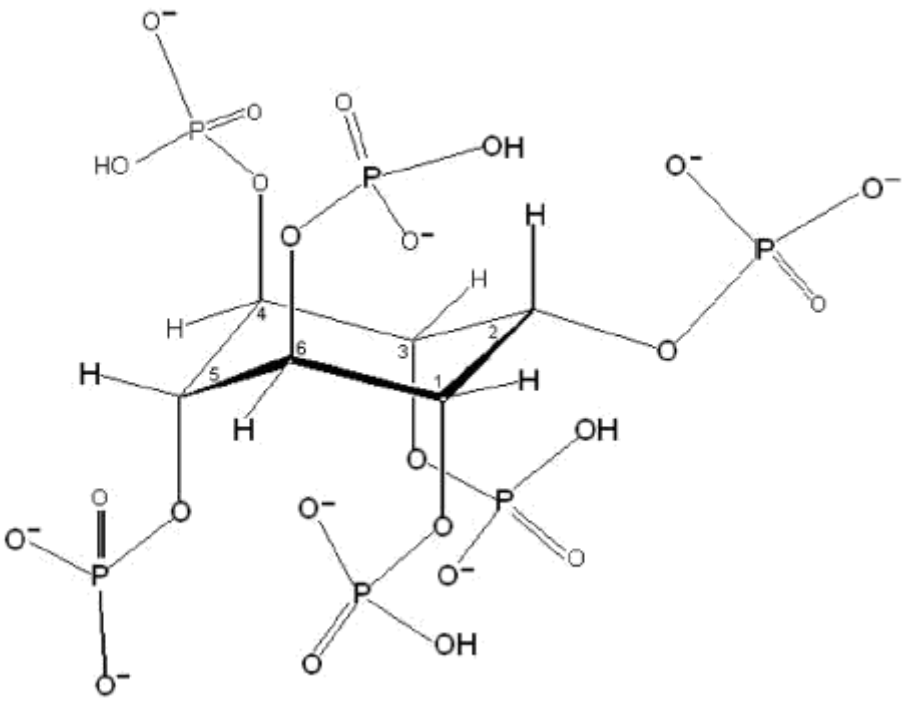


Figure 2: Structure of myo-inositol-1, 2, 3, 4, 5, 6,-hexakis phosphate from Schlemmer et al. (2009)

Phytases are enzymes which hydrolyse phytates to release free inositol and/ or intermediate compounds like esters of mono, bi, tri, tetrakis and pentakisphosphate inositol as well as phosphoric acid (Pandey et al., 2001; Konietzny and Greiner, 2002).

Phytases are found in plants and seeds and the intestine of some animals. Humans however have insignificant amounts of phytase activity in their intestine (Iqbal et al., 1994) so consuming a phytate rich diet results in phytate being undigested and P is not released for absorption, with other nutrients being immobilized by the formation of complexes.

The phytate content in cereals can be decreased by using traditional processing methods like fermentation. Fermentation can diminish the phytate content by up to 90%. This is due to the production of microbial phytase from the microbial fermentation culture which is either added as a starter culture or forms part of the natural microbiota found on the surface of the fermented food (Michaelsen et al., 2009). Endogenous phytases can be activated by fermentation, especially if low molecular weight acids like lactic acid are produced during the

fermentation because a pH of 6 or below produces the optimal activity for endogenous enzymes (Bohn et al., 2008).

2.10 Tannins and tannases

Phenolic compounds are a group of hundreds of molecules which are found in edible plants that have a benzenic ring substituted by at least one hydroxyl group. The number of phenolic rings they contain as well as the structural elements which bind these rings to each other allow for classification of these compounds into different groups (Rodríguez et al., 2009).

Phenolic compounds are classified into simple molecules like phenolic acids which have one aromatic structure, biphenols like ellagic acid, flavonoids which have 2-3 aromatic rings (Ignat et al., 2011) or polyphenols which have 12 to 16 rings. Tannins (condensed and hydrolysable), flavonoids, lignans, phenolic acids, stilbenes and are the main polyphenolic groups (Balasundram et al., 2006; D'Archivio et al., 2007).

Tannins are polyphenolic compounds with variable molecular weights (Aguilar-Z´arate et al., 2014) and are classified into 4 main groups: gallotannins, ellagitannins, condensed tannins and complex tannins (Khanbabaee and Ree, 2001) (Figure 3).When a galloyl or di-galloyl unit is esterified with a polyvalent alcohol or glucose core like tannic acid, it results in the formation of gallotannins which are easily hydrolysed by acid or alkali conditions or heat and are found in plants where tannic acid is acquired (Hagerman, 2002).

Ellagitannins forms the foundation of ellagic acid and are esters of hexa hydroxyl diphenic acid (HHDP) linked to a molecule of glucose (Aguilar-Z´arate et al., 2014).Condensed tannins are polymeric and oligomeric procyanthocyanidins and comprise of units of flavan-3-ol (catechin) or flavan -3-4 ol interconnected by carbon-carbon (C-C) bonds. Condensed tannins are not easily used as substrates by the tannase enzyme and the main components of these tannins are delphinidin and cyanidin which cause the sharp flavour of wine and fruit (Belmares et al., 2004; Ramirez-Coronel et al., 2004; Aguilar et al., 2007). Groups of gallotannnins or ellagitannins and an epicatechin or catechin make up complex tannins (Khanbabaee et al., 2001).

In human diets, tannins form complexes with proteins and metals which prevent the digestion and absorption of nutrients, thereby decreasing the nutritional value of foods (Chung et al., 1998).

Tannases are inducible enzymes produced by bacteria, yeast and fungi and have mostly been distinguished according to their activity on polyphenolic complexes, with a major role in degrading gallotannins (Knudson, 1913). *Aspergillus* spp are the most efficient commercial producers of tannase (Bhat et al., 1998). Tannase is membrane bound and found extracellularly in fungi, yeasts and bacteria. The optimum pH for tannase is 5.0-6.0 (Iibuchi et al., 1968; Iibuchi et al., 1972; Thomas and Murtaugh, 1985) while the optimal temperature is 35°C.

Bacteria like *Bacillus licheniformis* (Mondal and Pati, 2000) and many lactobacillus species (Osawa et al., 2000) also produce tannase. *Lactobacillus* species, *Bacillus* sp. and *Serratia* sp. have all been reported as tannase producers (Mondal et al., 2001; Pepi et al., 2010). The application of tannase in food and beverage processes is limited because of inadequate knowledge about its ideal expression and scale-up (Aguilar et al., 1999).

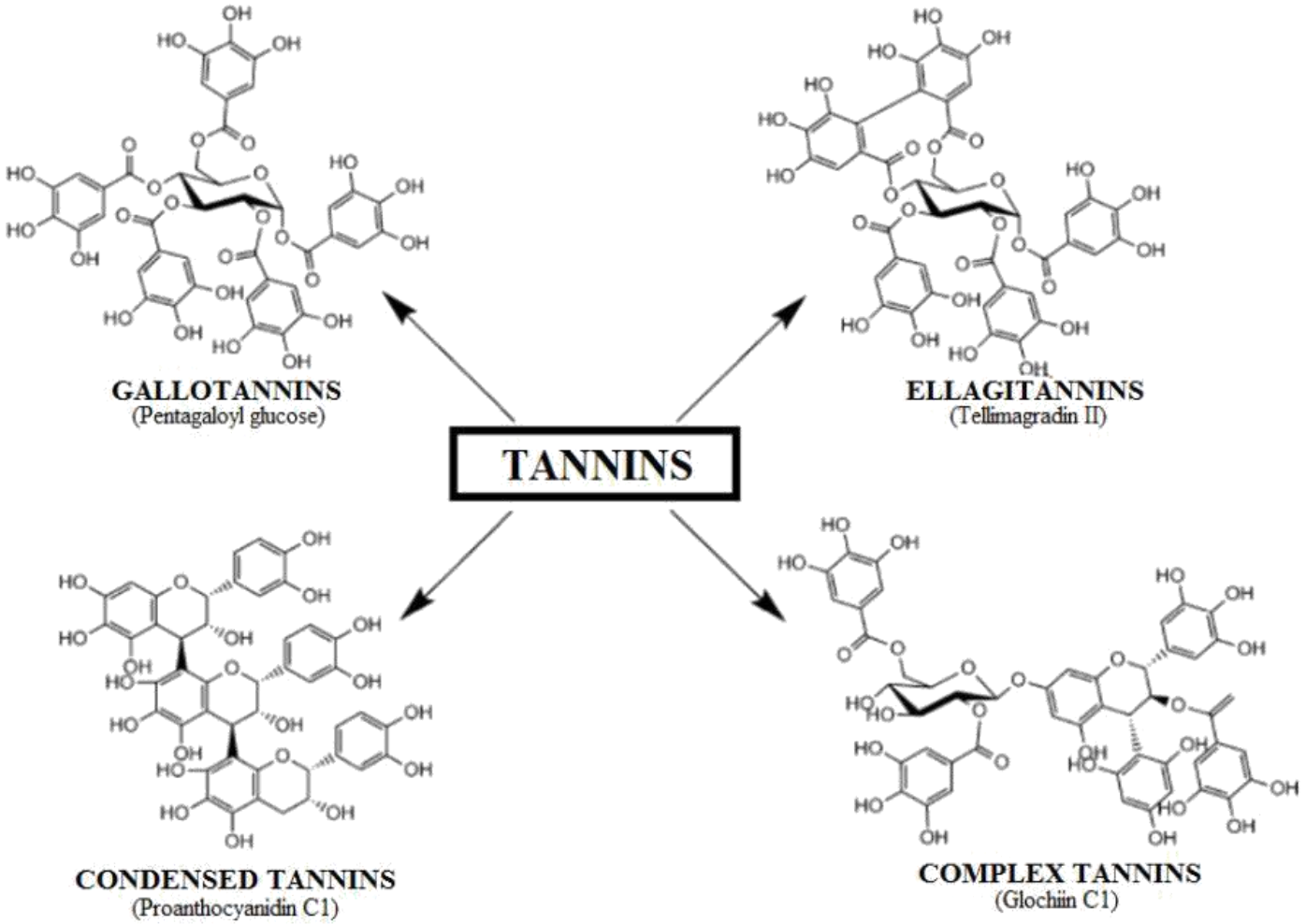


Figure 3: The structure of gallotannins, ellagitannins, condensed tannins and complex tannins from Aguilar-Z´arate et al. (2014).

2.11 Bioavailability

Bioavailability refers to the effectiveness of absorption of a compound which is consumed as well as its bioactive metabolites so that it can be delivered and used by target tissues (Failla and Chitchumronchokchai, 2005). It is difficult to calculate the effect which the total amount of micronutrients in a plant food will have on the micronutrient needs of a population. It is thus important to know what the amount of bioavailable micronutrients are when eaten in a common diet in order to determine what the nutritional result will be (Glahn et al.,1998).

Bioavailability is comprised of 3 steps, namely:

1) digesting and solubilizing elements in the gastro intestinal tract (GIT),
2) absorption of the element by intestinal cells and its transportation into circulation,
3) and assimilation from the circulation to the target or functional entity (Verhoeckx et al., 2015).

Bioavailability also encompasses bioaccessibility and bioactivity. Bioaccessibility is the amount of compound which is released from the food matrix within the GIT and is available for absorption by the intestine (Verhoeckx et al., 2015). Bioactivity is the transportation of the nutrient or bioactive compound to its target tissue as well as the biomolecular interaction, metabolism or biotransformation which can occur as well as the creation of biomarkers and induced physiological responses (Etcheverry et al., 2012).

2.12 Caco-2 (Adenocarcinoma colon) human cell line bioavailability studies

The food product, process or preparation all has an impact on the bioavailability of compounds from food (Wienk et al., 1999; van het Hof et al., 2000). The bioavailability of

minerals and trace elements from food is the fraction of the mineral which is absorbed and used by the body. In the small intestine, many components in the diet form insoluble or soluble complexes with minerals and elevate or diminish their availability for absorption. Knowledge of factors such as pH, solubility of minerals, and residence times at absorption sites which affect bioavailability of minerals and trace elements, will help in designing diets for susceptible groups of the population (Minekus et al., 1995).

In order to rank foods with regards to Fe bioavailability, a simple reproducible method to test bioavailability which mimics the *in vivo* condition in humans is essential. A 2-step *in vitro* digestion and measurement of soluble or dialyzable Fe is used to determine Fe availability (Miller et al., 1981). Dialyzability can identify the main differences in availability but it cannot calculate the degree of response (Fairweather-Tait et al., 2005; Fairweather-Tait et al., 2007).

In vitro digestion and Caco-2 collectively can predict bioavailability as well as the uptake into enterocytes and sometimes absorption (Sandberg, 2010).

2.13 Caco-2 model for iron uptake and transport

Caco-2 is derived from adenocarcinoma cells (Fogh et al., 1977) which spontaneously differentiate at confluence to acquire traits of small intestinal enterocytes and are therefore useful in order to study events which occur in the small intestine (Sandberg, 2010). This implies that Caco-2 cells provide a suitable way to study the mode of Fe absorption in the human intestine. The Caco-2 cell model uses a 2 step digestion process (Miller et al., 1981). The food is stomach digested with pepsin (1h, pH2, 37°C) and intestine digested with pancreatin and bile salts (2h, pH 6.5, 37°C) (Penugonda et al., 2018). The digesta is then incubated with the Caco-2 cells to become accessible cell uptake. The digesta is removed after a 4h and nutrients level in the digesta is measured.

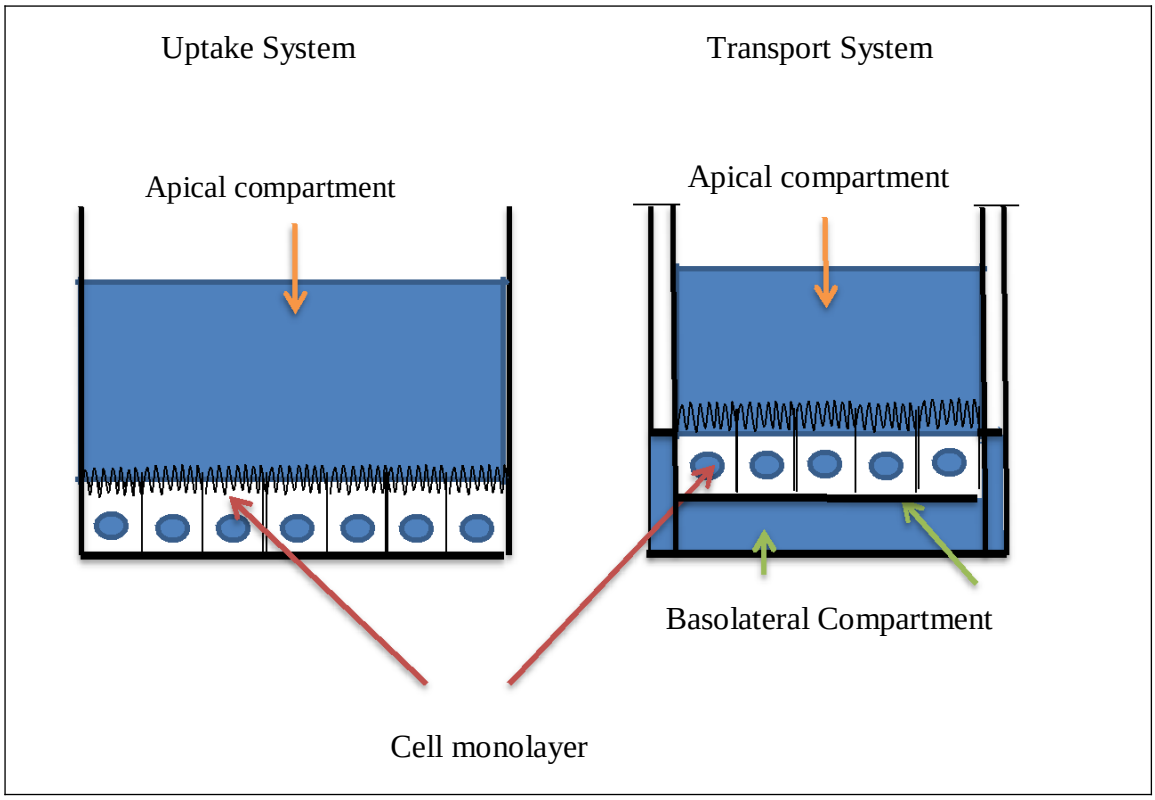


Figure 6: Caco-2 cell model for investigating the cellular uptake, metabolism and transport of Fe across cells. This model will be used to determine the uptake of Ca, Zn, and P during the current study on motoho. Modified from Failla and Chitchumronchokchai (2005).

2.14 Biofortification

Biofortification is the increase of bioavailable micronutrients of crops through plant breeding and genetic selection. These crops are consumed by the poor and can escalate the amount of micronutrients which are consumed by these target populations (Welch and Graham, 1999). Fortification and supplementation programmes require continual financial disbursements, unlike biofortification where the investment for plant breeding is upfront and micronutrient rich biofortified plants are provided to farmers with practically zero marginal costs (Bouis et al., 2017).The aim of biofortification is to increase the nutrient density in staple crops and/or increase their bioavailability by standard plant breeding or by using transgenic methods or a mixture of the two (Welch and Graham, 2005).

Biofortification is a strategy to address micronutrient malnutrition by plant breeding and organic approaches like micronutrient fertilizer in order to improve the concentration of key nutrients in principal foods (Bouis et al., 2011).

The role of biofortification is to decrease the mortality and morbidity rates in relation to micronutrient malnutrition and to increase food security and the quality of life for the poor populations living in developing countries by growing staple crops at low costs but with increased micronutrient bioavailability (Consultative Group on International Agricultural Research, 2002). This is different to industrial fortification and supplementation which depend on industrial processing and specialized channels of distribution or access to markets and health systems which are often not found in rural areas (Mayer et al., 2008).

The genetic selection of desired traits being placed into plant crops is how biofortification aims to increase the nutrient bioavailability of crops (Welsh and Graham, 2005). In developing countries plant foods are important components of the diet (Taylor and Taylor, 2011) with about 300 million people in Africa being reliant on sorghum as a staple food (Improving Food and Nutrition, 2015). Chemically induced mutations as well as genetic engineering have been used to biofortify sorghum (Taylor and Taylor, 2011). It could increase the nutritional status of the poor who have sparse dietary diversity, little or no access to commercially available products and marketed fortified foods, and only sporadic exposure to nutrient supplements (Taylor et al., 2012).

2.15 Fermentation

Fermentation is one of the oldest and most effective ways of food production and preservation. During fermentation, microorganisms, typically LAB or yeast, multiply and produce various enzymes like amylases, lipases and proteases which affect the viscosity, nutritional value and taste of products (Michaelsen et al., 2009). Spontaneous fermentation is initiated by the microorganisms inherent in the food but industrial fermentation makes use of defined starter cultures.

In food processing, fermentation has 5 functions:

1) Developing a wide range of aromas, textures and flavours in food and thereby enriching the human diet.

2) Preserving large amounts of food through the production of acetic, alcoholic, lactic and alkaline fermentations.

3) Biologically enriching food substrates with amino acids, essential fatty acids, vitamins and protein.

4) Detoxifying food.

5) A reduction in cooking times and also the energy requirements (Karovičová, 2007). Cereals are important substrates for fermented foods in Africa and Asia where energy and capital usage for food production and preservation is limited.

During food fermentations, enzymes and microorganisms result in desirable modifications and changes in texture and flavour of the food (Karovičová, 2007). In African countries, lactic acid fermentation of cereal products is widely practiced at the household level resulting in products like mahewu (Bvochora et al., 1999) and togwa (Lorri and Svanberg, 1995). Also, the low pH of these foods renders them safe from pathogenic bacteria (Charalampopoulos et al., 2002; Schnürer and Magnusson, 2005; Guarner et al., 2012). This is important for rural Africans who have the least access to clean water than any other developing area in the world (The United Nations Educational, Scientific and Cultural Organization, 2003).

Traditionally fermented products are indigenous to certain regions and have been developed by indigenous people using locally sourced raw materials and age-old techniques. In many developing countries, traditional methods are handed down from one generation to the next (Abegaz et al., 2002). Traditional fermentation methods are gathering increased interest as an essential part of food security strategies (van de Sande, 1997) and for commercial use.

Fermentation is a cheap method of preserving food as well as increasing the nutritional and sensory quality. Milk and cereal fermentations to produce beverages which promote health are indigenous to many Asian, European, South American, Middle Eastern and African states (Marsh et al., 2014). The functional beverage market globally is growing and consumers increasingly want foods to improve their health and decrease the possibility of illness.

Fermented milks like kefir from Western Europe and ymer from Denmark are well-liked functional beverages (Marsh et al., 2014).

2.16 Non-dairy fermented beverages

Fermented beverages made from cereals are popular in Africa. The natural microflora of the cereal (maize, sorghum, millet, rice, wheat, oats, barley and rye) is used for the fermentation (Marsh et al., 2014). Back-slopping (Ray, 2004) is a common practice in which a traditional fermentation is started by inoculating raw materials with a deposit from a preceding fermentation. However, the microbes used in these fermentations are not well characterized. The fermentation of an assortment of cereals like maize, wheat, rice, oats, millet or barley, is used to produce the beverage Boza, which is consumed in Turkey and Bulgaria (Akpinar-Bayizit et al., 2010). The chosen cereal is boiled, filtered and supplemented with a carbohydrate. The mixture is then spontaneously fermented or back-slopped. Boza is not commercialized and its microbial content is variable. It has been suggested that the most palatable drink is achieved by using *Leuconostoc meseteroides*, *Lb. confusus* and *Saccharomyces cerevisiae* (Zorba et al., 2003).

2.16.1 Kunan-zaki

Kunan-zaki is a Nigerian non-alcoholic fermented beverage which has been produced using a starter culture consisting of *Lb. lactis*, *Lb. fermentum* and *Lb. plantarum* (Agarry et al., 2010). This resulted in a product with better aroma, taste, acceptability and mineral bioavailability when compared to the control sample.

2.16.2 Gowé

Gowé is a malted fermented sorghum product which is consumed extensively consumed in Bénin (Michodjèhoun-Mestres et al., 2005). Vieira-Dalodé et al. (2008) produced gowé with individual LAB cultures of *Lb. fermentum Lo25* and *Weisella confusa L015*,

resulting in faster acidification of the substrate and inhibition of pathogenic growth as well as comparable sensorial scores compared to the traditional beverage.

2.16.3 Togwa

Togwa is a fermented beverage or gruel made from sorghum, millet, maize, cassava or combinations thereof (Mugula et al., 2001). Mugula et al. (2003) isolated *Lb. fermentum*, *Lb. brevis, Lb. cellobiosus, Pediococcus pentosaceus* and *W. confusa* from togwa.

2.16.4 Mahewu (amahewu)

Mahewu is a fermented maize beverage or gruel largely consumed by indigenous South African populations (Chelule et al., 2010) and is a source of energy for children and adults (Chelule et al., 2010). *Lactobacillus plantarum*, *Lb. delbrueckii*, *Lactococcus lactis* and *Streptococccus thermophiles* have all been used to manufacture mahewu (Odunfa and Adeyele, 1985; Holzapfel, 1989; Sanni, 1993; Steinkraus, 1995). More recently, Idowu et al (2016) used *Lb. brevis* as a starter culture in combination with *Sac. cerevisiae* to produce mahewu.

2.16.5 Motoho

Motoho is a traditionally fermented sorghum beverage which can be consumed by the whole family and is prepared daily or weekly. It has short shelf-life of roughly 5 days. Although little is known about the microbiological composition of motoho, Sakoane and Walsh (1988) assessed the safety of motoho as a weaning food and showed that motoho inhibited strains of *Escherichia coli*, *Shigella boydii*, and *Salmonella thypi* after 3 hours of fermentation suggesting that the acid produced during fermentation could have inhibited these bacteria due to the drop in pH of the product dropped from 7.2 to 6.4. Figure 4 shows motoho which has been traditionally produced as well as the modified motoho which was used as a control in the current study.



Figure 4: Traditionally prepared motoho. Image by S. Moodley©, CSIR, Pretoria, South Africa, 2015.

A **B** **C**

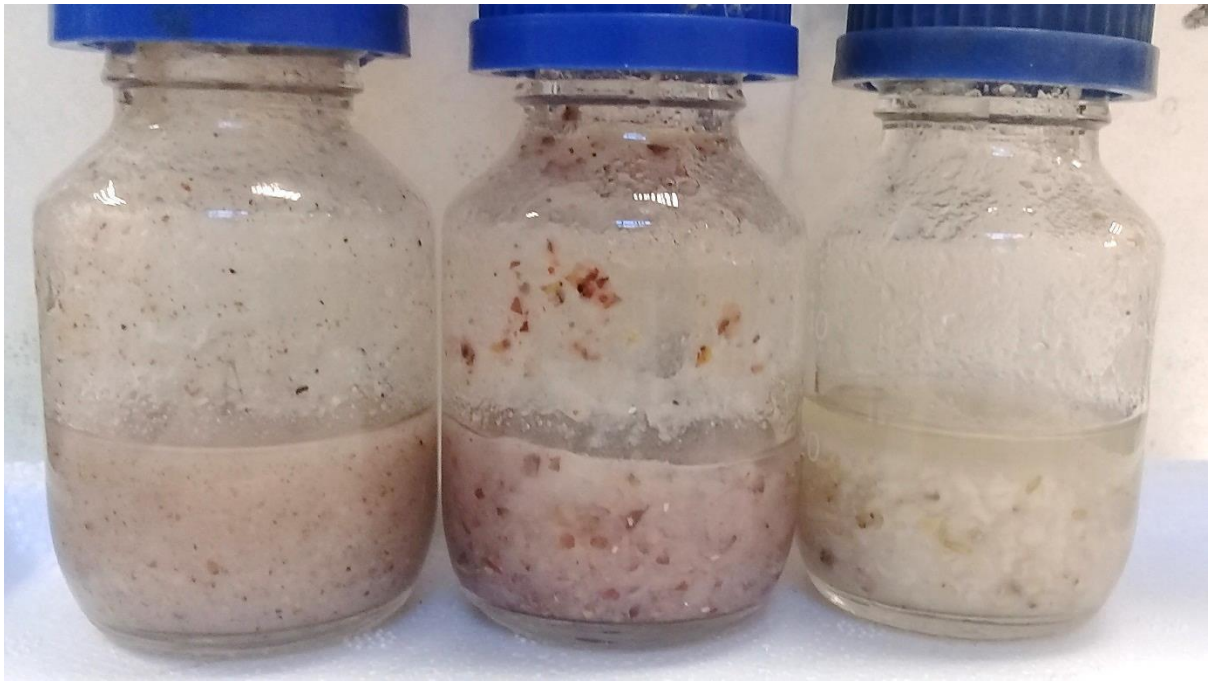


Figure 5: Motoho prepared on a laboratory scale using commercial sorghum (A) as the control and biofortified sorghum (B and C). Image by S. Moodley©, Council for Scientific and Industrial Research (CSIR, Pretoria, South Africa, 2018.

2.17 Aromatic compounds and their metabolic processes

The quantities of organoleptic active compounds is raw cereals is minimal and thus provide unpleasant flavours and odours (Zhou et al., 1999). Similarly, some phenolic compounds, present in the outer grain layers grain could contribute to astringency and bitterness (Heiniö et al., 2011). Fermentation has been proven to increase the textural and sensory qualities of liquid cereals. Studies using LAB starter cultures inoculated into cereals have focused on the resultant flavour and textural changes. Natural cultures have been replaced with defined starter cultures but still trying to maintain the sensorial characteristics.

Lactobacillus plantarum has been chosen for most of the work conducted on novel fermented liquid cereal products because it is robust when the pH is low (Charalampopoulos et al., 2002), thus *Lb. plantarum* is more beneficial when compared to other autochonous

microorganisms found on grains and it also produces pleasant "dairy" related flavours like acetaldehyde, acetoin, and diacetyl (Prado et al., 2008; Salmerón et al., 2015).

The flavour profile of fermented cereals is made up of aldehydes, ketones, esters, alcohols, organic acids and sugars (Mugula et al., 2003; Mukisa et al., 2012; Muyanja et al., 2012). Table 1 shows the different classes of volatile organic compounds and the sensory characteristics they produce.

Table 1: Volatile compound classes and their sensory characters. Modified from Fisher and Scott, 2001.

Compound Class	Sensory Character	Examples
Aldehydes	Fruity, green Oxidized, sweet	Acetaldehyde, hexanal Decanal, vanillin
Alcohols	Bitter, medicinal	Linalool, menthol
	Piney, caramel	α-Terpineol, maltol
Esters	Fruity	Ethyl acetate,
	Citrus	ethyl butyrate
		Geraniol acetate
Ketones	Butter, caramel	Diacetyl, furanones
Maillard	Brown, burnt, caramel,	Pyrazine(s), pyridine,
reaction products	earthy	furans
Phenolics	Medicinal, smokey	Phenol(s), guaiacols
Terpenoids	Citrus, piney	Limonene, pinene
	Citrus	Valencene

During food fermentations, flavour formation results from the accumulation of volatile and non-volatile aromatic compounds as well as from compounds which are related to taste. Alcohols, ketones, fatty acids, aldehydes, esters and sulphur compounds are responsible for aroma (Engels et al., 1997). The chemical precursors of these compounds are varied. Proteins, lipids and carbohydrates all deliver precursors for the conversion to aroma compounds. Functional metabolic pathways rather than single enzyme conversions are used for the formation of aroma compounds in LAB (Smid and Kleerebezem, 2014). The

contribution of LAB to volatile organic compound (VOC) production during the fermentation of cereals has not received much attention.

2.18 Starter cultures

In order to reproduce the desired traits of traditional beverages for mass production, appropriate starter cultures need to be selected. The microbes sourced from these beverages have been adapted to their environment over many years and will be able to function at the appropriate conditions like temperature, salt concentration (Marsh et al., 2014).

Strain selection also must ensure their correct balance of texture, speed of fermentation, the optimal amount of organic acids, aroma, flavour, vitamins and minerals (Marsh et al., 2014).

The use of definitive microbial strains influences the large-scale manufacture of fermented cereal products (Klaenhammer and Fitzgerald, 1994). The development and impact of starter cultures is of increased interest in order to standardize the fermentation; however the use of starter cultures by small-scale processing units is limited. The selection criteria for LAB cultures depend on the desired properties for the final product and the characteristics of the raw material (Soro-Yao et al., 2014). Lactic acid fermentation involves removing fermentable carbohydrates, O_2 consumption and organic acid formation with a drop in pH. An essential part of the fermentation of cereal-based products is the production of adequate amounts of organic acids and the decrease in pH to below 4.0 within a 24hr fermentation period (Hounhouigan et al., 1993, Hounhouigan et al., 1999; Annan et al., 2003; Viéira-Dalodé et al., 2007). The microbial antagonism of LAB could be due to the production of organic acids, H_2O_2, CO_2 ethanol and diacetyl either singly or in combination and also due to bacteriocins formation (De Vuyst and Vandamme, 1994). The shelf-life and microbial safety of fermented products is thus enhanced due to the rapid production of these compounds which could inhibit spoilage or pathogenic microorganisms (Omemu and Faniran, 2011; Okerere et al., 2012; Ekwem, 2014)

2.19 Problem Statement

Micronutrient malnutrition affects over 3 billion people, mostly women and children and infants of resource-deprived families (Kennedy et al., 2003). In many developing nations, micronutrient malnutrition continues to increase. Current intervention programs such as supplementation and food fortification have been unsustainable and ineffective in many countries (Darnton-Hill, 1999). Iron deficiency is the main cause of anaemia and affects 1.62 billion people globally (Benoist et al., 2008) while Zn deficiency results in 1.4% of all deaths worldwide and is also a global health issue (WHO, 2002). In sub-Saharan Africa, Zn and Fe deficiency are widespread in households who largely consume cereal based diets (Uusiku et al., 2010). Sorghum is a staple cereal in many parts of Asia, Africa and the semi-arid tropics globally (Duodu et al., 2003) with over 500 million people in in the semi-arid tropics consuming sorghum (Board on Science and Technology for International Development, 1996).Sorghum is high in phytates and sometimes tannins which further decrease the non-haem Fe and Zn bioavailability (Hunt, 2003).

Phytic acids and tannins in sorghum 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 such as Fe, Zn, Mg, Ca and K (Dvořáková, 1998), which decreases the bioavailability of these minerals (Kumar et al., 2010). Biofortification uses plant breeding and organic approaches to increase the concentration of key nutrients in principal foods, thereby targeting micronutrient malnutrition (Bouis et al., 2011).

Motoho is a non-alcoholic fermented sorghum beverage produced in Southern Africa by the Basotho (people of Lesotho). It is traditionally made by mixing sorghum meal with warm water to produce slurry. Tomoso (a traditional starter culture) is then added to the mixture which is allowed to ferment for 24h in summer or between 48-72h in winter. The fermented product is boiled and cooled before serving (Gadaga et al., 2013).

Motoho could however be used to address micronutrient malnutrition. Regular sorghum is used to manufacture motoho which is produced by spontaneous fermentation. This is an uncontrolled process that depends upon microorganisms originating from the raw material, utensils, or added as a starter culture (Nout and Motarjemi, 1997) and often leads to fermentation failure, unpredictable microbiological flora or delayed fermentations. Also, the length of and product quality resulting from a spontaneous fermentation are difficult to control and can result in products with a short shelf-life (Ogunbanwo et al., 2013).
This can be overcome by isolating and purifying the predominant organisms found in an acceptable product and using said organism as a starter culture to initiate the fermentation (Togo et al., 2002).

The first hypothesis of this study was that the use of a starter culture which comprised of a single or multiple species of LAB which were isolated during a previous study on motoho would decrease the variability of the end product. Motoho was produced on a small scale in the laboratory during prior research and certain LAB strains were isolated and identified to the genus level. These strains were used singly and in combination as starter cultures for the optimization of motoho production, in order to standardize the fermentation process.

The basis of hypothesis two is that biofortified products could increase the nutritional standing of the poor who have little variety in their diets, have limited or no access to commercially available products and fortified foods, and only intermittent exposure to nutrient supplements (Taylor et al., 2012). Therefore, hypothesis two of this study is that the nutritional value of motoho would be enhanced by using biofortified sorghum to manufacture motoho and in doing so motoho could be used to address the issue of micronutrient malnutrition.

The impact of this research could be the production of a beverage which may potentially assist in the fight to curb malnutrition in South Africa. This project will also serve to increase the existing body of knowledge on motoho since there is very little information currently on the optimization of motoho production.

2.20 Aims

The aims of this study are:

- To use selected starter cultures singly or in combination in order to standardize motoho production and quality.

- To determine the effect of fermentation on the nutritional quality of different varieties of motoho.

- To use in vitro digestibility Caco-2 cell assay to determine the mineral absorption of the different varieties of motoho.

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3 Sensory analysis and organic flavour compound production during the fermentation of motoho, a sorghum beverage

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Sensory analysis and organic flavour compound production during the fermentation of motoho, a sorghum beverage

Sanchia S. Moodley[1], Lucia Steenkamp[1] and Vincent M. Gray[2]

[1]CSIR Biosciences, PO Box 395, Pretoria, Gauteng 0001, South Africa

[2] Department of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg, 2050, South Africa

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Author note

All correspondence concerning this paper should be addressed to

Sanchia S. Moodley

CSIR Biosciences, PO Box 395, Pretoria, Gauteng 0001, South Africa

E-mail: sanchia.moodley@gmail.com

ScholarOne, 375 Greenbrier Drive, Charlottesville, VA, 22901

Abstract

Traditionally fermented products are developed by local inhabitants of a region and the production method is handed down through generations. These methods result in products with variable quality and a short shelf-life. Motoho is a traditionally produced fermented sorghum beverage indigenous to the Basotho people of Southern Africa. The current research aims to determine the effects of using a defined starter culture comprising of *Lactobacillus fermentum* and *Lactobacillus plantarum* compared to the traditional production, on the sensory qualities and shelf-life of motoho. Three different sorghums were used to produce batches A (traditional), J (modified) and M (modified) of motoho respectively. Samples were subjected to 4ºC and 37ºC storage for the shelf-life study while a sensory panel assessed the acceptability of each motoho variety. The total plate counts for A, J and M incubated at 4 ºC were 1 log cfu/ml, 1.48 log cfu/ml and 1.3 log cfu/ml on day 5 respectively. The counts for A, J and M incubated at 37ºC were 3.48 log cfu/ml, 3.18 log cfu/ml and 3.01 log cfu/ml on day 5 respectively. The largest amounts of ethyl and methyl esters were produced during the fermentation of J which could account for the preference in aroma and flavour by sensory panellists. Aldehydes were found in the fermentation of A while acetoin and butanediol were produced during the fermentation of M. Panellists also preferred the appearance of J but the mouth feel of M. The results from this study could be applied during upscaling of the production of motoho.

Keywords

Motoho, volatile organic compounds, sensory analysis, starter cultures, shelf-life, sorghum fermentation

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Traditionally fermented products are indigenous to certain regions and have been developed by indigenous people using locally sourced raw materials and age-old techniques. In many developing countries, traditional methods are handed down from one generation to the next (Abegaz, Beyene, Langsrud and Narvhus, 2002) and is acquiring increased interest as an essential part of food security strategies (van de Sande, 1997) and for commercial use. Fermentation is a cheap method of preserving food as well as increasing the nutritional and sensory quality. Milk and cereal fermentations to produce beverages which promote health are indigenous to many Asian, European, South American, Middle Eastern and African states (Marsh, Hill, Ross and Cotter, 2014). Cereals are important substrates for fermented foods in Africa and Asia where energy and capital usage for food production and preservation is limited (Karovičová, 2007). In the food industry, the functional beverage market globally is growing and consumers increasingly want foods to improve their well-being and decrease the risk of disease (Marsh et al. 2014). Motoho is a traditionally fermented sorghum beverage produced by the Basotho people of Southern Africa and can be consumed by the whole family. It is prepared daily or weekly and has a short shelf-life of roughly 3 days. Regular sorghum is used to manufacture motoho by spontaneous fermentation which is an uncontrolled process that depends upon microorganisms which originate from the sorghum, utensils or added as a traditional starter culture (Nout & Motarjemi, 1997). This results in products with variable quality and sensory attributes (Chaves-López et al., 2014). This can be overcome by isolating and purifying the predominant organisms found in an acceptable product and using said organism as a starter culture to initiate the fermentation (Togo, Feresu and Mutukumira, 2002). Previous research on motoho included isolating and characterizing lactic acid bacteria (LAB) namely *Lactobacillus fermentum* and *Lb. plantarum* which were selected as potential starter cultures for the optimization process of motoho. *Lactobacillus plantarum* was also identified in Bushera and in many other fermented and cooked products (Kunene, Geornaras, von Holy and Hastings, 2000) as well as in maize products like ogi (Akinrele, 1970; Odunfa & Adeyele, 1985; Johansson, Sanni, Lönner and Molin, 1995; Steinkraus, 1995) and is dominant towards the culmination of many spontaneous cereal fermentations (Nout, 1980; Olasupo, Olukoya and Odunfa,1997) possibly due to high acid tolerance (Akinrele, 1970; Hounhouigan, Nout, Nago, Houben and Rombouts, 1993).

Lactobacillus fermentum was isolated during the fermentation of a Sudanese sorghum flatbread called kisra (Mohammed, Steenson and Kirleis,1991), a Beninese fermented maize dough called mawe (Hounhouigan et al. 1993) and also in a Beninese fermented and malted sorghum food called gowe (Vieira-Dalodé et al., 2007).

It was assumed that the use of starter cultures to produce motoho could decrease the variability of the end product. In order to reproduce the desired traits of traditional beverages for mass production, appropriate starter cultures need to be selected. The microbes sourced from these beverages have been adapted to their environment over many years and will be able to function at the appropriate conditions like temperature and salt concentration (Marsh et al. 2014). Strain selection also must ensure the correct balance of texture, speed of fermentation, the optimal amount of organic acids, aroma, flavour, vitamins and minerals of the final product (Marsh et al. 2014). The aim of this research was to determine the effect of the selected LAB strains on the shelf-life and sensory acceptability of motoho produced with 3 varieties of sorghum and by using traditional and modified methods of production. This research could contribute to the body of knowledge regarding motoho as well as to assist in future upscaling and possible commercialization of the product.

2 Materials and Methods

2.1 Sorghum

Commercial pure grain sorghum (King Korn Mabele, King Food Corporation, Potchefstroom, South Africa) was purchased locally and used for the control motoho.

Two varieties (Rakodzi and SC Smile) of brown, high tannin sorghums were used to produce modified motoho and were obtained from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Matapos Research Station, Bulawayo, Zimbabwe.

2.2 Microbial strains

Lactobacillus fermentum was isolated and identified during previous research on motoho and stored at -20°C in glycerol. *Lactobacillus plantarum* ATCC culture was purchased from Microbiologics (St. Cloud, Minnesota, U.S.A).

To obtain pure colonies these bacteria were sub-cultured thrice onto MRS (de Man, Rogosa and Sharpe agar) (Oxoid, Basingstoke, UK) agar which was incubated for 48h at 37˚C.

Pure bacterial cultures were inoculated into 10ml of MRS broth (Merck, Darmstadt, Germany) and incubated for 24h at 37˚C, centrifuged at 4500rpm (Beckman Coulter, Brea, California, U.S.A) for 10min and the supernatant was discarded. The cell pellets were re-suspended in 0.85% NaCl solution to produce a stock of 10^7 cfu/ml. For the starter culture for the control fermentation, commercial sorghum was added to regular tap water (1:10 w/v). Five grams of commercial brown sugar was then and the mixture was incubated for 24h at 30˚C. *Lactobacillus plantarum* and *Lb. fermentum* (1:1 v/v) were used as the starter culture for the production of the modified motoho (J and M).

2.3 Production of motoho

The production of both the traditional motoho (A) as well as the motoho produced using Rakodzi sorghum (J) and SC Smile sorghum (M) is represented in Figure. 1.

2.4 Microbial analyses

Five millilitres of each motoho batch were diluted into 45ml of sterile Maximum Recovery Diluent (MRD) (1g bacteriological peptone (Oxoid) and 8.5g NaCl per litre of distilled water). Total plate counts (TPC), LAB counts and yeast and mould counts were determined by surface spreading 0.1ml of serially diluted motoho in duplicate. Tryptone Soya Agar (Merck) plates were used to test for all viable mesophilic aerobic organisms and MRS agar (Merck) was used for LAB detection. Both TSA and MRS plates were incubated anaerobically for 48h at 30˚C. Yeasts and moulds were quantified on Potato Dextrose Agar (PDA) (Merck) which was incubated for 120h at 25˚C. The MPN method was used to test for the presence of *Escherichia coli* (*E.coli*) by using test tubes containing Lauryl Tryptose Broth (LTB) (Oxoid). The tubes were incubated at 37°C for 24h and checked for the presence of gas production.

2.5 Shelf-life

Shelf-life studies of batches A, J and M were conducted in triplicate at 4ºC and 37ºC for a period of 5 days. Samples were tested in duplicate daily for pH, *E.coli* and TPC.

2.6 Volatile organic compounds

A composite of the 10 replicates of A, J and M were made respectively. Each composite was tested in triplicate for Volatile organic compounds (VOC's).

Five hundred milligrams (mg) of each sample was added to 2 ml of 99.9% dichloromethane (DCM) (Sigma Aldrich, St. Louis, Missouri, and U.S.A), sonicated at room temperature for 10 min and then filtered.

The analysis was conducted on a Leco (St. Joseph, Michigan, United States) GCxGC-TOF low resolution mass spectrophotometer equipped with a BPX-5 column type with a length of 28.838 m, internal diameter 250µm, and film thickness of 0.25 µm with a maximum temperature of 360 ºC. Samples (2µL) were injected and a splitless mode was activated. The flow rate was 1.4 ml/min at constant flow while the front inlet temperature was 280 ºC. The initial oven temperature of 50 ºC was maintained for 4 min and ramped at 15 ºC/min to 300 ºC and held at this temperature for 10 min. The transfer line temperature was 300 ºC. The aroma compounds of motoho were determined using a mass range of between 35 to 450 m/z. Probability based matching with mass spectra from the NIST (NIST Ver. 2.1 2009 Mass Spectra Library, Gaithersburg, Maryland, USA) library was used for further identification.

2.7 Hedonic test

An untrained acceptability panel in the Food Science department of the University of Johannesburg in South Africa was used to evaluate the sensory properties of the three varieties of motoho.

The appearance, aroma, mouth feel, flavour and overall like or dislike of the 3 motoho samples were assessed by using a 9-point hedonic scale to rate the products (Stone & Siddel, 1985) (9= like extremely, 8= like very much, 7= like moderately, 6= like slightly, 5= neither like nor dislike, 4= dislike slightly, 3= dislike moderately, 2= dislike very much, 1= dislike extremely).

3 Statistical analyses

Data was analyzed by one way Analysis of Variance (ANOVA) (Fisher, 1918) with a significance level of $p < 0.05$ and principal component analysis (PCA) using the PRINCOMP procedure from the SAS statistical software.

4 Results

4.1 Microbiological counts of produced motoho and shelf-life

Microbiological counts for the produced motoho were all <10cfu/ml for TPC, LAB counts and yeasts and moulds. Figure 2 shows the mean TPC counts (log cfu/ml) during the 5 day shelf-life study. No counts were detected on the first day of the shelf-life but counts increased to 3.07 log cfu/ml at day 2 for A (4ºC) and then decreased to 2.6, 1.3 and 1 log cfu/ml at days 3,4 and 5 respectively. Counts increased for A (37ºC) to 3.15 log cfu/ml at day 2 , then decreased to 2.49 and 2.3cfu/ml on days 3 and 4 respectively, before increasing to 3.48 log cfu/ml at day 5. Counts of 1.56 log cfu/ml and 1.91 log cfu/ml were obtained for J at 4ºC and 37ºC respectively at day 2. While the counts for J (4ºC) ranged from 2.18, 1.7 and 1.48 log cfu/ml over the 5 day shelf-life period, the counts for J (37 ºC) increased to 4 log cfu/ml on day 3 then decreased to 2.7 log cfu/ml on day 4 before increasing to 3.18 log cfu/ml on day 5. Counts of 3.16 log cfu/ml and 3.34 log cfu/ml were achieved for M at 4ºC and 37ºC respectively at day 2. The counts for M (4ºC) then steadily declined over the 5 day shelf-life to 2.11, 1.48 and 1.3 log cfu/ml while the counts for M (37ºC) increased to 3.61 log cfu/ml on day 3, declined slightly to 2.79 log cfu/ml on day 4 and then increased to 3.01 log cfu/ml on day 5. *Escherichia coli* was absent throughout the 5 day shelf-life study for all the batches of motoho.

4.2 Volatile organic compounds

The number and relative percentages of the volatile organic compounds (VOC's) found in the 3 different varieties of motoho are represented in Table 1.

A total of 18 VOC's whose area were greater than or equal to 3%, were identified in this study. They were largely categorized into 7 groups which included an alcohol (1), aldehydes (2), esters (10), hydrocarbon (2), alkane (1), fatty acid (1) and ketone (1). Out of the 18 volatile compounds, five unique compounds occurred in A and J respectively while 4 occurred in M.

The remaining 4 compounds were common between A, J and M. The ester 9, 12-Octadecadienoic acid, methyl ester, (E, E) - and organochloride phosphinous chloride, (chloromethyl) (1, 1-dimethylethyl) - were common to A, J and M while the hydrocarbon 4-Decene, 2, 2-dimethyl-, (Z) - was common to A and J. The ester Undecanoic acid, methyl ester was found in both J and M.

Esters accounted for 60.34% of the total volatile organic compounds found in A, with Cyclopropanepentanoic acid, 2-undecyl-, methyl ester, trans- being the most predominant.

An organochloride (9.68%), aldehydes (19.64%) and a hydrocarbon (10.34%) were also found.

The VOC's of J were for the most part made up of esters (75.10%) with 9-Octadecenoic acid (Z)-, methyl ester being the major ester. Other organic compounds found in J were a fatty acid (5.19%), an alkane (7.30%), a hydrocarbon (4.47%) and an organochloride (7.94%). In M, 55.49% of the VOC's primarily constituted esters (55.49%) with 9, 12-Octadecadienoic acid, methyl ester, (E, E) - being the most predominant. An alcohol made up 25% of the total while a ketone and an organochloride made up 9.3% and 10.21% respectively.

4.3 Hedonic test

The results from the hedonic test of the 3 varieties of motoho are represented in Table 2.

There were significant differences ($p < 0.05$) between the motoho varieties in terms of aroma and flavor with panelists preferring the aroma and flavor of J. The appearance, mouth feel and overall differences between the 3 varieties of motoho were not significant ($p > 0.05$).

Figure 3 shows the Principal component analysis (PCA) of the sensory properties of the 3 different varieties of motoho. The variable system included flavor, mouth feel, appearance and aroma. Principal component 1 (PC1) accounts for 61.24% of variance across the samples while PC2 accounts for 16.86% of the variance. On PC1, all 4 variables have an almost equal weight, but flavor has the highest weight and is therefore the most important part of that component. For PC2, aroma is the most important part, with both appearance and mouth feel having a negative or reducing effect. Along PC1 (Figure3), J and M appear towards the right, indicating high levels in flavor while A appears to the left of PC1, showing a high negative intensity in flavor. The results along PC2 indicate a more even distribution of A, J and M both towards the right and left of PC2. However A appears more towards the right of PC2, showing a negative intensity in aroma.

5 Discussion

The flavour of food is subject to the balance of the volatile compounds which are found in foods or by those which are made during the production of foods (Linton & Wright, 1993).

Ogunremi, Agrawal and Sanni (2015) developed a cereal based fermented functional food which was a combination of pearl millet, wheat and white and red sorghum and used *Pichia kudriavzevii* OG32 as the probiotic.

Esters accounted for 32.37% and acids accounted for 32.21% of the VOC's in this functional food and are mainly responsible for the pleasant aromatic notes adding to fruity and floral odours of fermented foods (Chen & Xu, 2010; Moreira et al., 2011; Parkouda et al., 2011). The smallest proportions of VOC's were alkanes and aromatic compounds which made up 6.02% of the total. In the motoho batches A, J and M, no acids were detected. However, methyl and ethyl esters were the predominant VOC's activity after the product has been made (Kutyauripo, Parawira, Tinofa, Kudita and Ndengu, 2009). This was also apparent in the shelf-life study of motoho where cell counts of the motoho samples incubated at 37ºC continued to increase over the course of the 5 day shelf-life study. Overall, the microbiological counts for A, J and M which were incubated at 37ºC increased while the counts for A, J and M samples incubated at 4ºC decreased over the 5 day shelf-life study. Microbial populations decreased when samples were pasteurized and refrigerated and a shelf-life of 24 days was achieved with kunan-zaki. LAB is thermoduric which allows them to survive at pasteurization temperatures with the highest area percentage of 55.9% esters found in J, followed by M (25.84%) and A (23.51%). Similarly to the results obtained by Ogunremi et al. (2015), one of the smallest concentrations of VOC's of motoho were alkanes (5.43%).

Esters were predominant in the production of pito and burukutu, fermented sorghum alcoholic beverages from Nigeria (Onyenekwe, Erhabor, and Akande, 2016). Ethyl esters of straight chain fatty acids are common in cheese and could be formed enzymatically by bacteria when ethanol is condensed with a carboxylic acid (Curioni & Bosset, 2002).

They impart a fruitiness to Italian type cheeses but they could also impart a fruity off-flavour if found in high concentrations (Liu, Holland, and Crow, 2004; Thierry, Maillard, Richoux and Lortal, 2006). While methyl esters are formed by the methanolysis of acyl-CoA which is made during the synthesis or degradation of fatty acids and affect the sensory properties of a variety of fermented foods (Perestrelo, Fernandes, Albuquerque, Marques, and Câmara 2016). Lyumugabe, Songa, Wathelet and Thonart (2013) found esters in sorghum beer and Liu et al. (2004) found 9 esters in fermented stinky tofu. Batch J of motoho had the highest concentration of esters, which could explain the preference of the flavour of J by the panellists conducting the sensory analysis.

Butanediol is an alkanol which was predominant in the fermentation of pito and burukutu (Onyenekwe et al. 2016) as well as in batch M of motoho.

Alkanols impart an alcohol like aroma and property which is often linked to indigenous beverages, consequently giving beer its warm mouth feel. Low alcohol concentrations impart fruity-type aromas in foods while increased concentrations are responsible for “hot” aromas which are not desirable to the consumer (Saberi, Cliff and Van Vuuren, 2012). Ethanol was not identified in pito or burukutu, sorghum beer (Lyumugabe et al. 2013) or all 3 batches of motoho. This is in contrast to Campbell-Platt (1994) who found ethanol to be the main source of alcohols present in fermented cereals. The lack of ethanol could be the result of the quick conversion of ethanol to the intermediary products by alcohol dehydrogenase which is found in fermenting microorganisms. The fermentation conditions and time of fermentation could also contribute to the lack of ethanol (Onyenekwe et al. 2016). Aldehydes and ketones are typically found in alcoholic beverages to which they contribute objectionable organoleptic properties (Wardencki, Sowiński and Curyło, 2003).

The degradation of lipids and amino acids during microbial fermentation results in ketone formation which strongly impacts the odour of foods (Owens, Allagheny, Kipping and Ames, 1997). Pito and burukutu contained 3 aldehydes, namely 3-methyl butanal, acetylaldehyde, 2-methyl and propanal which may have impacted the odour. Aldehydes (9, 12-Octadecadienal and Octanal, 7-methoxy-3, 7-dimethyl-) were found in Batch A of motoho while acetoin, a ketone was found in batch M.

Muyanja, Narvhus, Treimo and Langsrud (2003), also discovered acetoin during the fermentation of bushera, a non-alcoholic traditionally fermented sorghum beverage from Uganda. Diacetyl and acetoin are the result of LAB using pyruvate and citrate (Anaya, 1996; Narvhus, Østeraas, Mutukumira and Abrahamsen, 1998). Acetoin-like products in cereals are associated with bacterial instead of yeast-dominated flora (Halm, Lillie, Sørensen and Jakobsen, 1993). This is consistent with the fermentation of batch M of motoho since LAB starter cultures were used for the fermentation. The lack of aldehydes or ketones in batch J could explain the preference of the aroma of this batch. Out of the 3 batches of motoho, only J contained a fatty acid (1, E-11, Z-13-Octadecatriene). Fatty acids can impart cheesy, fruity, rancid and fatty notes and are important in establishing the equilibrium in wines because they prevent the hydrolysis of the corresponding ester (Torrens et al., 2008) and their presence is important in the aroma complexity. The presence of the fatty acid in batch J of motoho could have also contributed to the preferred aroma.

Traditionally fermented African cereal products have a limited shelf-life of between 3-5 days (Aka, Konan, Fokou, Dje and Bonfoh, 2014) which can be attributed to unpleasant off flavours or over souring which results from the sustained microbial and are hence found in heat treated samples. The microbes present are constrained by low storage temperatures (Osuntogun & Aboaba, 2004). This could account for the lower microbial numbers which were found during the storage of motoho at 4ºC.The rapid decrease in the shelf-life of many African beverages is extensively recognized and is of great interest (Odunfa & Oyeyiola, 1985; Efiuvwevwere & Akona, 1995). High microbial loads and diverse microorganisms are the main causes of spoilage encountered by producers and consumers of preservative free and unpasteurised traditionally made products (Efiuvwevwere & Akona, 1995). Sodium benzoate is commonly used as a preservative for foods and soft drinks (Lennerz et al., 2015) and was also used during the production of motoho. The maximum acceptable microbial load for fermented foods at any given point during the products shelf-life is 10^5 cfu/g (Stannard, 1997). This is in line with the counts obtained for the shelf-life of motoho with the highest counts of 10^4 cfu/ml obtained by J stored at 37ºC on day 3 of the shelf-life study.

This implies that for all the samples in the study, microbial counts were well below the recommended range and that the motoho produced in this study has shown to have an extended shelf-life. Use of sodium benzoate during the production of motoho could have been responsible for the low cell counts, especially during the storage of motoho at 37ºC. Many traditionally fermented African cereal based products spoil rapidly and are unsatisfactory to consumers within 1 to 4 days of being produced (Nout, 1980). However the results of this study show acceptable microbial loads even at day 5 of storage.

6 Conclusion

The shelf-life study showed that the motoho microbial counts were dependent on temperature, irrespective of the traditional or defined starter cultures which were used for motoho production. However, even at an accelerated temperature, the microbial counts were below the recommended microbial load for beverages, even at day 5 of the shelf-life. Further shelf-life studies could be conducted past the 5 day period in order to determine the effect of a longer storage time on the product quality. Although it is recommended that motoho is generally consumed within 3 days, the product will last even at day 5.

Overall, a sensory panel preferred motoho which was produced using the defined starter culture and brown high tannin sorghum Rakodzi. The high percentage of esters found during the production of this motoho could have accounted for the preference in aroma and flavour. This sorghum, along with the selected LAB strains could be used for further research on motoho in order to upscale the production and possibly commercialize the product as it has shown to be a highly favourable product with an extended shelf-life.

Author Contributions

Miss Sanchia Moodley conducted all the research for this article as well as writing the content for the article. She is the primary author.

Dr Lucia Steenkamp has edited the article and has provided direction for the research methods. She is also a co-supervisor for this research.

Prof Vincent Gray has edited the article and has provided guidance for the research methods. He is also the academic supervisor.

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Ethical statements

Ethical review: This study was approved by the University of the Witwatersrand Human Research Ethics Committee (Medical).

Conflict of Interest: The authors declare that they do not have any conflict of interest.

Informed Consent: Written informed consent was obtained from all participants in this study.

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Table 1: Percentage area of the volatile organic compounds found in 3 different varieties of motoho (A, J, M). Data represented are the means of triplicate values. – is not detected.

Compound type		% Area A	% Area J	% Area M
Aldehyde	9,12-Octadecadienal	3.88	-	-
	Octanal, 7-methoxy-3,7-dimethyl-	3.77	-	-
Ketone	Acetoin	-	-	5.43
Esters	9,12-Octadecadienoic acid, methyl ester, (E,E)-	4.71	9.92	9.57
	Acetic acid, bis[(trimethylsilyl)oxyl]-, trimethylsilyl ester	3.74	-	-
	Cyclopropanepentanoic acid, 2-undecyl-, methyl ester, trans-	6.4	-	-
	Undecanoic acid, methyl ester	4.86	-	6.5
	Pentadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	3.8		
	9-Octadecenoic acid (Z)-, methyl ester	-	38.38	-
	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	-	3.69	-
	Tridecanoic acid, methyl ester	-	3.91	-
	11-Octadecenoic acid, methyl ester	-	-	12.88
	Dodecanoic acid, methyl ester	-	-	3.39
Hydrocarbon	4-Decene, 2,2-dimethyl-, (Z)-	4.03	3.33	-
	Phosphinous chloride, (chloromethyl)(1,1-dimethylethyl)-	3.77	5.91	5.95
Fatty Acid	1,E-11,Z-13-Octadecatriene	-	3.86	-
Alkane	3,5-Dithiahexanol 5,5-dioxide	-	5.43	-
Alcohol	2,3-Butanediol, [S-(R*,R*)]-	-	-	14.56

Table 2: Sensory analysis of 3 different varieties of motoho. Data represented are means and standard deviations with n=65

Sample	Aroma	Flavour	Appearance	Mouth feel	Overall quality
A	5.80 ± 2.10	5.91 ± 2.17	6.17 ± 2.0	5.66 ± 1.76	6.00 ± 1.94
J	6.74 ± 1.86	6.82 ± 1.81	6.42 ± 1.9	5.97 ± 2.17	6.50 ± 1.98
M	6.69 ± 1.55	6.74 ± 1.92	6.32 ± 1.7	6.00 ± 1.93	6.69 ± 1.72

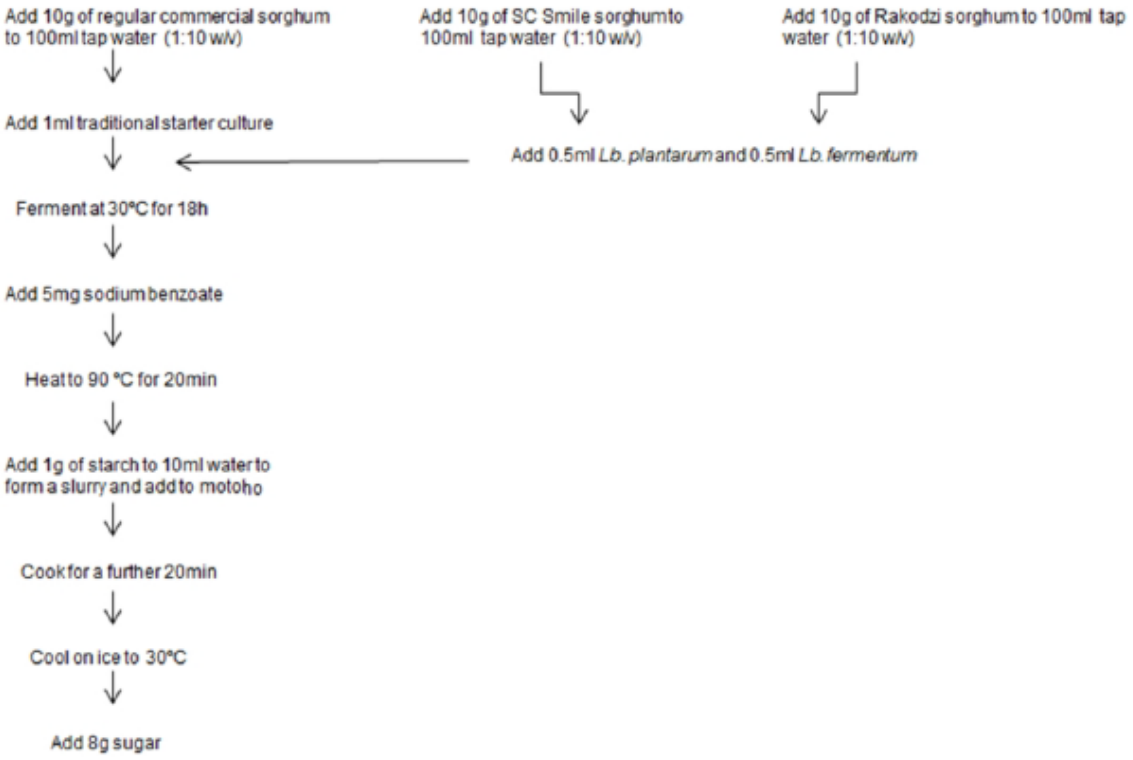
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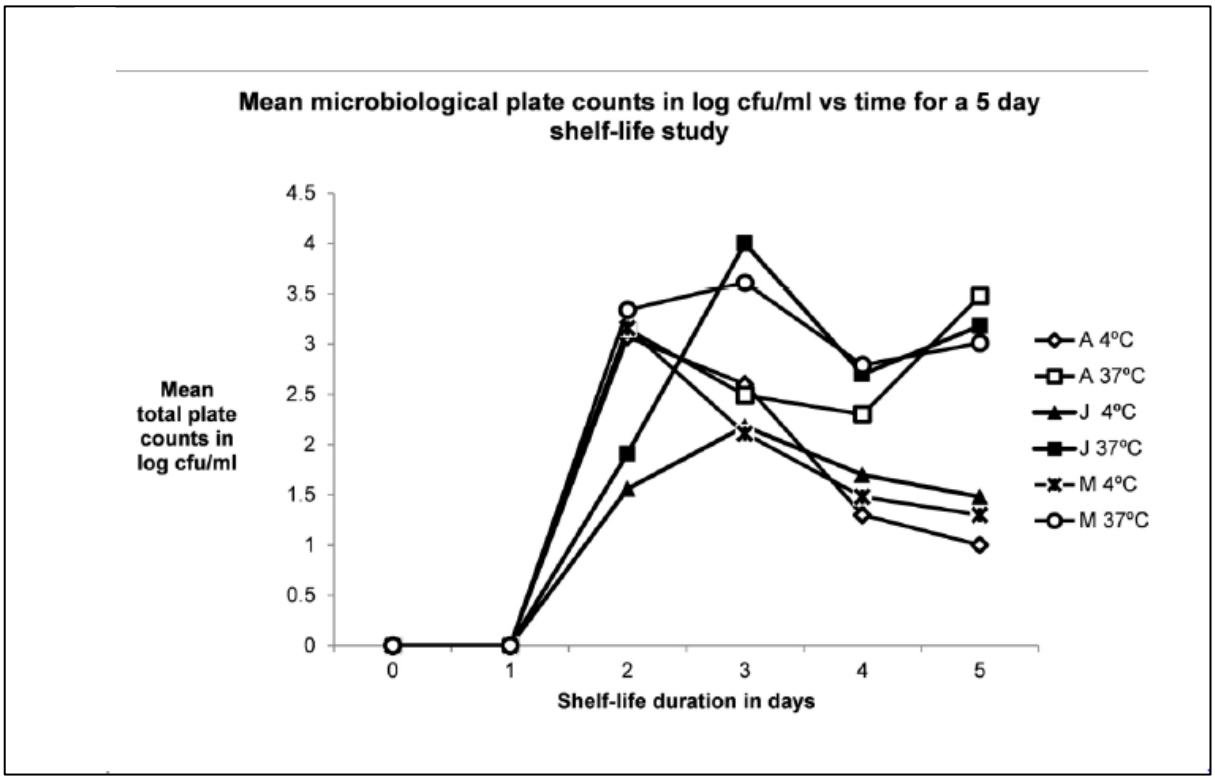
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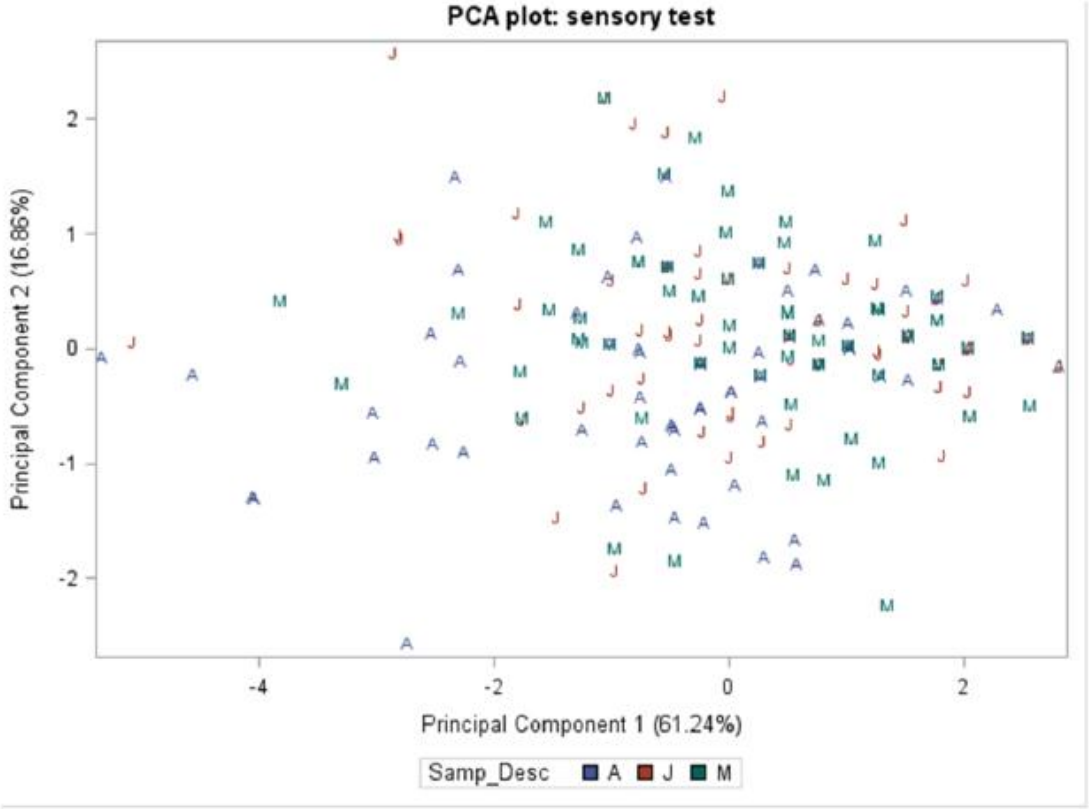
Figure 1 Flow chart outlining the production of the traditional motoho (A) and variations of this method (J and M) using defined starter cultures. Ten replicates of each motoho were produced.

Figure 2: Total bacterial plate counts (log cfu/ml) obtained for 3 varieties of motoho incubated at 4ºC and 37ºC during a 5 day shelf-life study. N=3

Figure 3: Principal component analysis of the sensory analysis of 3 different varieties of motoho using 65 panellists who rated the motoho using a 9 point hedonic scale. The traditional motoho is represented by A and was produced using spontaneous fermentation while J and M represent the modified motoho produced with starter cultures.


Add 10g of regular commercial sorghum to 100ml tap water (1:10 w/v)
Add 10g of SC Smile sorghum to 100ml tap water (1:10 w/v)
Add 10g of Rakodzi sorghum to 100ml tap water (1:10 w/v)
Add 1ml traditional starter culture
Add 0.5ml Lb. plantarum and 0.5ml Lb. fermentum
Ferment at 30°C for 18h
Add 5mg sodium benzoate
Heat to 90 °C for 20min
Add 1g of starch to 10ml water to form a slurry and add to motoho
Cook for a further 20min
Cool on ice to 30°C
Add 8g sugar


Mean microbiological plate counts in log cfu/ml vs time for a 5 day shelf-life study
Mean total plate counts in log cfu/ml
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
Shelf-life duration in days
A 4ºC
A 37ºC
J 4ºC
J 37ºC
M 4ºC
M 37ºC


PCA plot: sensory test
Principal Component 2 (16.86%)
2
1
0
-1
-2
-4
-2
0
2
Principal Component 1 (61.24%)
Samp_Desc
A
J
M

4 Impact of fermentation of motoho, a fermented Southern African sorghum beverage, on the enzyme activity, polyphenolic content, mineral content and uptake using the Caco-2 cell assay

Title: Impact of fermentation of motoho, a fermented Southern African sorghum beverage on the enzyme activity, polyphenolic content, mineral content and uptake using the Caco-2 cell assay.

Choice of journal/section: Journal of Food Science and Nutrition

*Sanchia S. Moodley[1], Lucia Steenkamp[1], Angelique Botha[3], Sophia M. Linsky[3] and Vincent M. Gray[2]

[1]CSIR Biosciences, PO Box 395, Pretoria, Gauteng 0001, South Africa

[2] Department of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg 2050, South Africa

[3] National Metrology Institute of South Africa , Private Bag X34, Lynnwood Ridge, Pretoria, 0040, South Africa

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*Author note

All correspondence concerning this paper should be addressed to

Sanchia S. Moodley

CSIR Biosciences, PO Box 395, Pretoria, Gauteng 0001, South Africa

E-mail: sanchia.moodley@gmail.com

Abstract

Motoho, a traditionally fermented sorghum food from Southern Africa was produced using four sorghum varieties as well as a traditional and defined starter cultures which comprised *Lactobacillus fermentum* and *Lactobacillus plantarum* in order to assess the impact of motoho fermentation on the tannin and phytate contents of sorghum. Sixteen motoho variations were screened using Inductively Coupled Plasma Sectorfield Mass Spectrometer and values for iron (28.87-45.91mg/kg^{-1}), zinc (17.7-26.6291mg/kg-^{1}), phosphorous (2386.09-3894.95 mg/kg^{-1}), calcium (107.63-385.57mg/kg^{-1})and magnesium (2386.09-3894.95 mg/kg-^{1}) content. Only the motoho variants showing the highest mineral contents were selected for further analysis. Compared to the raw sorghums, the tannin contents of the selected motoho were reduced by up to 94%. However, even though endogenous phytase was measured during motoho production the values were low (0.26-0.44U/ml) and the phytate contents were reduced by up to 9.3 %. The mineral bioavailability of iron, zinc, calcium and magnesium of the selected motoho varieties were conducted using Caco-2 cell uptake assays. The iron (0.20-0.51%), zinc (0.43-0.44%), magnesium (0.87-1.28%) and calcium (10.47-26.58%) uptake percentages could not meet the recommended daily intake for infants up to 12 months. Different pre-processing methods such as decortication, soaking and germination have been shown to activate endogenous phytates. These methods could be employed during further research on motoho in order to decrease the phytate content and thereby improve the mineral bioavailability of motoho.

Keywords: motoho, phytate, tannin, enzymes, mineral bioavailability, Caco-2

Introduction

Over 3 billion people worldwide, predominantly women, children and infants of resource-deprived families are affected by micronutrient malnutrition (Kennedy, Nantel and Shetty, 2003). In many developing nations, micronutrient malnutrition is increasing and current intervention programs such as supplementation and food fortification are unsustainable and ineffective (Darton-Hill, 1999). Globally, over 1.62 billion people have an iron (Fe)deficiency, predisposing them to anaemia (Benoist, McLean, Egll and Cogswell, 2008) while zinc (Zn) deficiency is the cause of 1.4% of all deaths worldwide (World Health Organization, 2002). In sub-Saharan Africa, Zn and Fe deficiencies are prevalent in households who consume mainly cereal based diets (Uusiku, Oelofse, Duodu, Bester, and Faber, 2010).

Sorghum is a staple cereal in many parts of Asia, Africa and the semi-arid tropics globally (Duodu, Taylor, Belton and Hamaker 2003), with over 500 million people in more than 30 of these countries consuming sorghum (Board on Science and Technology for International Development, 1996). Some varieties of sorghum contain anti-nutrients like tannins and phytates (Schons, Battestin and Macedo, 2012) which decrease mineral bioavailability (Kumar, Sinha, Makkar, and Becker 2010) by forming complexes with proteins (Hagerman & Butler, 1978) in the case of tannins or by forming insoluble phytate-mineral complexes (Dvořáková, 1998). However, fermentation has been shown to enhance the bioavailability of minerals and vitamins in foods (Altay, Karbancıoglu-Güler, Daskaya-Dikmen and Heperkan, 2013; Hwang, Kim, Moon, Yang and Kim, 2017).Traditional fermentation methods are also garnering increased interest as an essential part of food security strategies (van de Sande, 1997) and are seen as a cheap method of increasing the nutritional value of food (Marsh, Hill, Ross and Cotter, 2014).

Although cereal beverages are poorly studied, they are popular in Africa, often being used as a weaning food for children and as a high-energy supplement (Marsh et al. 2014). They are also an important dietary component for many people in developing countries where they form an essential part of the local customs and traditions (Borresen, Henderson, Kumar, Weir and Ryan, 2012; Chilton, Burton and Reid, 2015; Kanwar, 2016; Narzary, Brahma, Brahma and Das, 2016).

Motoho is one such beverage which is produced in Southern Africa by the Basotho (people of Lesotho). It is traditionally made by mixing sorghum meal with warm water to produce a slurry and is fermented by using Tomoso (a traditional starter culture) over a 24hr period in summer or between 48-72hr in winter. The fermented product is boiled and cooled before serving (Gadaga, Lehohla and Ntuli, 2013).

The present study sought to determine what the effect would be on the micronutrient, polyphenolic and phytic acid contents of motoho if it was produced with various modifications of the traditional method of processing. We also sought to determine what the result would be on the bioaccessibility and bioavailability of the micronutrients of motoho.

2 Materials and Methods

2.1 Chemicals

All chemicals, unless otherwise stated were from Sigma, St. Louis, Missouri, U.S.A

2.2 Sorghum

Commercial pure grain sorghum (King Korn Mabele, King Food Corporation, Potchefstroom, South Africa) was purchased locally and used for the control motoho.

Two varieties (Rakodzi and SC Smile) of brown, high tannin sorghums and two varieties (SV2 and Macia) of white, tannin free sorghums were used to produce modified motoho and were obtained from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Matapos Research Station, Bulawayo, Zimbabwe.

2.3 Microbial strains

Lactobacillus fermentum was isolated during previous research on motoho and stored at -20ºC in glycerol. *Lb. plantarum* ATCC culture was purchased from Microbiologics (St. Cloud, Minnesota, U.S.A complete. To obtain pure colonies these bacteria were subcultured thrice onto MRS (Oxoid, Basingstoke, UK) agar which was incubated for 48h at 37˚C.

Pure bacterial cultures were inoculated into 10ml of MRS broth (Merck, Darmstadt, Germany) and incubated for 24h at 37˚C, centrifuged at 4500 rpm (Beckman Coulter, Brea, California, U.S.A) for 10 min and the supernatant was discarded.

The cell pellets were re-suspended in 0.85% NaCl solution to produce a stock of 10^7 cfu/ml. The starter culture for the control fermentation was produced by adding 10g sorghum to 100ml water (1:10 w/v) and 5g brown sugar and the slurry was incubated for 24hr at 30˚C.

2.4 Production of motoho

Motoho was produced by using 4 variations outlined in Table 1, of the control method (Figure 1) Table 1 outlines the sorghum variety and starter culture used to produce the different motoho varieties.

The produced motoho beverages were freeze-dried (SIM International FD5 Series Freeze Dryer) at ≤ -50°C for 50-60 hr before being used for further analysis.

2.5 Mineral analysis

For Fe, calcium (Ca), phosphorous (P), magnesium (Mg) and Zn determination, approximately 0.5 g of freeze-dried motoho was microwave digested (Milestone ETHOS UP, Italy) using 8 mL of concentrated nitric acid (HNO_3) (69%, TraceSELECT, Honeywell Fluka, Italy)double distilled with a DuoPUR sub-boiling distillation system (Milestone, Sorisole, Italy) and 40 µL of hydrogen peroxide (H_2O_2) (30%, TraceSELECT, Honeywell Fluka, Italy).The concentrations of Fe, Ca, P, Mg and Zn were analyzed using an inductively coupled plasma sectorfield mass spectrometer (ICP-SFMS) (ThermoFinnigan MAT ELEMENT2, Thermo Scientific, Waltham, Massachusetts) according to the method of Kayodé, Linnemann, Hounhouigan, Nout and van Boekel (2006). Only the motoho varieties showing the highest mineral contents were subjected to further testing of phytase, tannase, tannins, phytates and absorption studies using Caco-2 cells.

2.6 Phytase assay

The phytase assay was performed on the fermented sorghum slurry according to the Enzymatic Assay of phytase (EC 3.1.3.26) from Sigma Aldrich.

2.7 Tannase assay

Tannase was assayed according to the method of Mondal, Banerjee, Jana and Pati (2001) on the fermented sorghum slurry.

2.7 Phytate content

The method of Haug and Lantzsch (1983) was used to determine the phytate content of freeze-dried motoho samples colorimetrically. Phytate phosphorous, in concentrations of 200-5000ug/ml was used as a standard and the phytate concentration was expressed as g Kg^{-1}.

2.8 Tannin high- throughput vanillin assay

Tannin content was determined according to the high throughput vanillin assay by Herald, Gadgil, Perumal, Bean and Wilson (2014). Tannins were extracted from freeze-dried motoho samples according to the method of Price, Scoyoc and Butler (1978). Each extract was centrifuged at 13000 rpm for 2min and 30µl of the supernatant or standard were aliquoted into appropriate wells of a 96 well plate (Thermofisher Scientific, Waltham, Massachusetts, U.S.A). Vanillin HCl (equal parts of 2% vanillin in methanol with 8% HCL in methanol) was prepared daily and added to one set of the extract while 4% HCL in methanol was added to the other set of extracts. The solutions were incubated at 30^{o}C for 20min and the absorbance read at 500nm on a Hidex Sense microplate reader (Separation Scientific, Honeydew, Johannesburg, R.S.A).The absorbance of the control samples with 4% HCL added was subtracted from the corresponding vanillin containing samples. Catechin was used as the standard in the construction of the standard curve. Tannins were expressed as catechin equivalents (CE) g Kg^{-1} in concentrations of 0.032 – 500ug/ml.

2.9 Caco-2 cell culture assay

2.9.1 Caco-2 cell passage

Adenocarcinoma colon (Caco-2) cells were obtained from Celltonex (Celltonex, Robindale, Randburg, South Africa) and cultured in HyClone (GE Healthcare Life Sciences, South Logan, Utah, United States) Dulbecco's Modified Eagle Medium (DMEM) with high glucose, L-glutamine and sodium pyruvate which contained 10% fetal bovine serum (FBS)(Hyclone) and 1% penicillin-streptomycin-neomycin (Thermofisher Scientific, Waltham, Massachusetts, U.S.A). The cells were grown in 75 cm^2 flasks at 37 °C with constant humidity in a 5% CO_2 incubator. The medium was changed every second day. The confluent cells were rinsed off the bottom of the flask with 0.25% trypsin–EDTA (HyClone) and centrifuged at 900 rcf for 2min.

Cells for the assay were seeded in 6-well culture plates with 0.4µm polycarbonate membrane inserts (AEC-Amersham, Kyalami, Midrand, South Africa) at 2×10^5 cells per well for 21 days and the medium was changed every second day. All experiments were conducted between the 15th-17th passages.

2.9.2 Caco-2 in-vitro digestion

The bioaccessibility of motoho was determined by using *in-vitro* digestion to simulate human digestion according to the method of Penugonda, Fiorentino, Alavi and Lindshield (2018) with slight modifications. One gram of each motoho variant was suspended in sterile distilled water (1:10 w/v).The pH of the sorghum samples were lowered to 2.0 with 1M HCL. Upon acidification 2ml of pepsin solution (40mg/ml in 0.1M $NaHCO_3$ was added and the final volume was made up to 40ml using Hanks Balanced Salt Solution (HBSS) (Hyclone). The samples were incubated in the dark for 1h at 37°C and 85rpm.

The gastric phase was ended by increasing the pH of the digesta to 6.0 using 1M $NaHCO_3$ and the samples were placed on ice. Two millilitres of lipase (5mg/ml made up with 0.1M $NaHCO_3$) and pancreatin (10mg/ml made up with $NaHCO_3$) as well as 3ml of bile extract (40mg/ml made up with 0.1M $NaHCO_3$) were added to the digesta and the pH was increased to 6.5 using 1M NaOH.

The sample volumes were then adjusted to 50ml using HBSS and incubated in the dark for 2h at 37ºC and 85rpm. After digestion, 10ml of each sample was centrifuged at 4500rpm for 45min. Iron and zinc contents of the supernatants were measured using ICP-SFMS as outlined in 2.5.

2.9.3 Caco-2 absorption assay

The supernatants (2.9.3) were mixed in equal parts with the same medium used to culture the cells, except only 2% FBS was added instead of 10% (Kruger et al., 2013). The transepithelial/endothelial electrical resistance (TEER) readings were measured before and after the absorption assay. Only wells which showed TEER readings of >250ohm were used for the absorption assay. Each well of the 6-well plates were inoculated with 0.5ml digest: medium mixture and incubated at 37 °C at 300rpm in an orbital shaker for 4h. The digest: medium mixture was collected separately from the apical and basolateral compartments of the 6-well plates. Iron and Zn contents in a blank (media only), the soluable mineral fraction, the apical and basolateral soluble mineral fractions (digest: medium mixture) were calculated using SFMS. The cellular retention of minerals (µg) was calculated

as the difference between in the mineral contents of the cells which were incubated with soluable mineral fraction and the cells which were incubated with a blank (media only).

Retention percentages and transport percentages were calculated as follows according to Camara, Barbera, Amaro and Farre (2007):

Retention (%) = 100 · R/C, where R = mineral retention (µg mineral/well), and C = soluble mineral content added (µg).

Transport (%) = 100 · T/C, where T = difference between mineral amounts in the basal chamber and transport buffer (blank) (µg mineral/well), and C = soluble mineral content added (µg).

Uptake= retention plus transport (µg). Bioaccessibility = 100 x Y/Z where Y= the element content of the bioaccessible fraction and Z = total mineral content (Hemalatha, Platel and Srinivasan, 2007).

2.10 Statistical analysis

Data was analysed by one way Analysis of Variance (ANOVA) (Fisher, 1919) with a significance level of $p < 0.05$.SAS statistical software was used for the statistical analysis.

3 Results

Figures 2 and 3 represent the results from ICP-SFMS which show the concentrations of Fe and Zn as well as Mg, P and Ca of the different motoho varieties respectively. Batch A of motoho had the highest level of Ca with 385.57mg/Kg-1 while N had the lowest concentration of 107.63 mg/Kg-1. Batch M of motoho had the highest P and Mg concentrations of 3894.96 and 1768.20 mg/Kg-1 respectively. Batch N of motoho had the lowest concentrations for P (2386.09mg/Kg-1) and Mg (1071.81mg/Kg-1). The highest Fe levels were found in A (45.91mg/Kg-1) while the lowest levels were found in N (28.87mg/Kg-1). Batch J of motoho had a Zn concentration of 26.62mg/Kg-1, which was the highest overall concentration while C had the lowest overall concentration of Zn (17.77mg/Kg-1). The Fe and Zn concentrations of each motoho variety were much lower when compared to the P, Mg and Ca values. The P values obtained for each motoho batch were the highest overall, with Mg and Ca being the next highest overall values respectively.

The lowest values obtained were Fe and Zn respectively. Based on the ICP-SFMS values, motoho varieties A, J and M were chosen for tannin, tannase, phytate and phytase determination as well as the Caco2 absorption assay.

Table 2 outlines the phytase, phytate, tannin and tannase concentrations of motoho varieties A, J and M as well as the phytate and tannin concentrations of the raw commercial sorghum and the Rakodzi and SC Smile sorghum flours. The highest tannase concentration was found during the fermentation of A, which also had the lowest tannin concentration. Out of the sorghum flours SC Smile had the highest tannin levels while the regular commercial sorghum had the lowest levels. The phytase and tannase were measured at the end of the 18h fermentation of the motoho slurry and hence no values were measured for the raw sorghum varieties.

The regular commercial sorghum had the highest phytate levels (2836 g/Kg-1) and its derivative motoho A showed the largest reduction in phytate content of 9.3% after fermentation. Rakodzi had a lower initial concentrations of phytate,(1776.67 g/Kg-^{1}) when compared to the regular sorghum. Their resultant motoho product J also showed a decrease in the phytate concentrations after fermentation with J showing a 1.05% decrease and M a 0.21% decrease after fermentation. Batch J of motoho had the lowest concentration of phytate (1758.05 g/Kg-1) even though the highest concentration of the phytase enzyme of 0.26U/ml was found during the fermentation of batch M.

The soluability percentage, retention, transport and uptake assays from the Caco-2 of all three motoho varieties are represented in Table 3.The highest mineral soluability percentage for both calcium and iron was shown by J while A showed the highest values for magnesium and zinc. Phosphorous values, even though detected in the initial samples were not represented for the Caco-2 assay because no results could be detected for retention, transport, and uptake. The highest uptake percentage for calcium (26.58%), magnesium (1.28%) and iron (0.51%) were shown by J while M showed a marginally higher uptake percentage of zinc (0.44%) compared to A and J (both 0.43%). There were no significant differences ($p>0.05$) for Ca, Mg, Feand Zn in terms of retention, transport and uptake for all motoho varieties.

Discussion

Fermented foods are recognized as being “healthy’ especially in societies where they are produced in the traditional manner, adding to their market potential (Marsh et al., 2014). There is a need for studies to confirm these health benefits which are conferred to consumers, especially because fermented cereals are frequently consumed in Africa and can contain high mineral contents(Marsh et al., 2014), thereby providing an option for targeting micronutrient malnutrition. During the current study, motoho was produced using different varieties of sorghum which can be affected by anti-nutrients like tannins and phytates (Léder, 2004).

Tannins form complexes with proteins and metals preventing the digestion and absorption of nutrients and decreasing the nutritional value of foods (Chung, Wong, Wei, Huang and Lin, 1998) while phytic acid strongly binds to Fe, Zn, Mg and Ca and prevents their absorption (Astley and Finglas. 2016).

The present research focused on the effect of the tannin and phytate contents of different motoho varieties as well as the tannase and phytase enzymes produced during motoho fermentation and also how these polyphenolic compounds and enzymes would influence bioaccessibility and bioavailability of the minerals of the different motoho varieties. Iron and Zn contents have been known to differ substantially between sorghum cultivars (Kruger et al., 2013).

Badi (2004) showed the total P content of 2 sorghum cultivars, Wad Ahmed, a high tannin cultivar and Tabat, a low tannin cultivar, to be 303mg/100g and 283.3mg/100g respectively while Deosthale and Belvady (1978) showed a range of P in sorghum of 388mg/100g to 756mg/100g. Similar P results were obtained in motoho (2386.09-3894.95 mg/kg-1).

The Ca content obtained for motoho varieties (107.63- 385.57 mg/kg-1) was within the range of Ca obtained for injera, (4.75mg/100g)(Mohammed, Ahmed, M and Babiker, 2011) while the Mg content of the motoho varieties ranged from 1071.81-1768.20mg/kg-1, similar to Oboh and Amusan (2009) who found levels of 112.9mg/100g in fermented sorghum gruels, similar to those obtained in motoho.

Motoho showed Fe and Zn values of 28.87-45.91 mg/kg-^{1} and 17.17-26.62mg/kg-^{1} respectively and these results concurred with what was previously found for fermented sorghum which ranged from 3.95 mg/100g (Mohammed et al. 2011) to 90mg/100g, (Oboh and Amusan, 2009) for Fe and 0.64mg/100g (Mohammed et al. 2011) to 2.1mg/100g (Oboh and Amusan, 2009) for Zn.

Wu et al. (2016) found that genotype and temperature significantly impacted the tannin content of the uncooked sorghum grains of six different sorghum genotypes. Cooking the sorghum greatly reduced tannin content by roughly 87% in the IS8525 sorghum porridge and a 30-76% reduction was seen in the other 5 genotypes (Wu et al., 2016) These results concur with and could explain the decrease in tannin after fermentation and cooking of the motoho varieties during which the tannin was decreased by 94.18% and 82.28% and 85.28% for motoho varieties A and J and M respectively.

There was a progressive and significant decrease in the phytic acid content observed during the fermentation Safra, Ahmer and Fetarita sorghums (Rahman and Osman, 2011). Values for phytic acid were initially between 369.1-276.3g/100g but decreased to 50.6mg/100g for Safra, 51.8mg/g for Ahmer and 54.6mg/100g for Fatarita (Rahman and Osman, 2011). Fermentation has been shown to cause a decrease in phytic acid in opaque sorghum beer (Kayodé, Hounhouigan and Nout, 2007) pearl millet (Mohamed, Amro, Mashier and Elfadil, 2007) and sorghum gruels (Towo, Matuschek and Svanberg, 2006). These results correspond to that obtained during the fermentation of motoho where phytic acid values decreased from between 2836-1776 g/kg-^{1} to 2572-1758g/kg-^{1}. The results obtained for all batches of motoho showed no significant differences ($P \geq 0.05$) could be found in the phytate content of the raw sorghum and resultant motoho batches.

Anastasio et al. (2010) showed the production of 0.71U/ml of phytase by *Lb. plantarum* HS. Slightly lower phytase values (0.26 – 0.44 u/ml) were obtained during the fermentation of motoho. Since phytases are affected by pH, temperature, and substrate specificity, amongst other variables, these could all account for the lower values obtained during motoho fermentation. The low microbial phytase activity and subsequent low phytate reduction in the production of motoho could also be accounted for by the deactivation of endogenous cereal enzymes during autoclaving of the raw sorghum flours. Ayed and Hamdi (2002) showed that *Lb. plantarum* was capable of producing 6U/ml tannase under optimal fermentation conditions which included growth on minimal media with 2 g glucose l^{-1} and 7 g tannic acid l^{-1} at pH 6 and 37 ◦C under anaerobic conditions. The tannase produced during motoho fermentation was lower with values of 0.67-1.34U/ml being obtained, quite possibly due to sub-optimal growth conditions during the production of motoho.

The phytate: mineral ratios can be used to predict the effects of phytate on mineral bioavailability (Ma et al., 2005).For phytate: Fe molar ratios <1, the inhibitory effect of phytate on Fe absorption is diminished (Mohite, Chaudhari, Ingale and Mahajan, 2013). The phytate: Fe molar ratios were 4.36-

6.89 in the present study on motoho and it can therefore be assumed that the phytate could have had an inhibitory effect on Fe absorption. A phytate to Ca molar ratio of >0.2 signifies compromised levels of Ca absorption (Sandberg, 2002).

This was apparent in motoho where phytate: Ca molar ratios of 0.4-0.77 were obtained. The critical molar ratio above which phytate inhibits Mg absorption is still unknown (Mohite et al. 2013). Hence no molar ratios are represented for phytate: Mg in motoho.

The molar ratios of phytate: Zn for motoho ranged from 6.5 for J, 11.8 for A and 11.10 for M. Saha, Weaver and Mason (1994) showed that the critical phytate: Zn molar ratio above which the Zn availability is considerably decreased is ≥ 10-15. This implies that for the motoho varieties A and M, Zn availability was decreased. However, no significant differences ($p>0.05$) were found in the uptake percentages of A, J or M of motoho and similar uptake percentages were found in each of the three motoho varieties.

The uptake percentages for Fe and Zn from different varieties of sorghum were found to be 0.76-0.98% and 1.21-1.89% respectively during a study conducted by Kruger et al. (2013). These values are higher than the uptakes percentages obtained during the current study on motoho where values of 0.20-0.55% were obtained for Fe and 0.43-0.44% for Zn.

Complementary foods made from millets, sorghum and maize have low bioavailability due to their high mineral inhibitor content and also because of a low content of enhancers such as proteins and ascorbic acid. Most African foods have high tannin and phytate levels which strongly inhibit the absorption of Fe and Zn (Gabaza, Muchuweti, Vandamme and Raes, 2017). According to the World Health Organization (2002), the daily Fe and Zn requirements are 9.3 and 3 mg per day respectively for infants between 9-11 months in age. Complementary foods should contribute between 75-100% of the total Fe and Zn daily requirements (Gabaza et al. 2017). The daily Mg and Ca requirements for children aged 0-12 months are 26-54 mg and 300- 400mg respectively (Joint, FAO & World Health Organization. 2005). However, as eluded to by Gibson, Ferguson and Lehrfeld (1998) and confirmed by Gibbs et al. (2011), most complementary foods, even those with moderate bioavailability cannot provide sufficient Zn or Fe. The results from the bioavailability studies on motoho also confirm that motoho is unable to provide any of the RDA for Fe, Zn, Ca or Mg.

Dephytinization, by adding exogenous enzymes or by activating naturally occurring endogenous phytases could increase the mineral bioavailability in infant cereal foods (Hurrell, 2004).

Frontela, Scarino, Ferruzza, Ros and Martínez (2009) by using Zn uptake and retention data of three commercial infant cereals, showed that phytate reduced the Zn bioavailability, as has been previously reported (Han, Failla,, Hill, Morris and Smith Jr, 1994; Hansen, Sandström and Lönnerdal, 1996).

The production of many millet and African sorghum porridges under standardized conditions has resulted in a decrease of over 50% of phytic acid after spontaneous fermentation (Kayodé, Mertz, Guyot, Brat and Mouquet-Rivier, 2013; Kruger, Taylor and Oelofse, 2012; Mouquet-Rivier et al., 2008; Proietti, Mantovani, Mouquet-Rivier and Guyot, 2013; Tou et al., 2007; Towo, Matuschek, and Svanberg, 2006). However, Gabaza et al. (2018) showed a general lack of phytic acid reduction during the traditional fermentation of finger millet sour porridges with only one variety of porridge showing a decrease in phytic acid. These results also concur with the production of motoho where phytate was decreased minimally after fermentation and cooking even though endogenous phytase was detected during the fermentation of motoho. Pre-processing factors like decortication, soaking and germination can all influence the reduction of phytic acid after fermentation (Kayodé et al. 2013). These processes were not used during the production of motoho and could account for the relatively low decrease in phytate after fermentation and cooking.

Values of 0.3-4% Fe bioaccessibility has been reported after the fermentation of numerous fermented African cereals (Matuschek, Towo and Svanberg, 2001; Towo et al. 2006; Greffeuille et al., 2011; Baye, Mouquet-Rivier, Icard-Vernière, Rochette and Guyot, 2013; Baye , Mouquet-Rivier, Icard-Vernière, Picq and Guyot ,2014; Baye, Guyot, Icard-Vernière, Rochette and Mouquet-Rivier, 2015). The values obtained for Fe bioaccessibility for the motoho products were slightly lowers (0.49-0.67%). These low values could be attributed to the phytic acid as IP6 content not being degraded to lower inositol after motoho production since a greater than 90% rate of degradation is needed in order to ensure a two fold increase in the bioavailability of Fe (Hurrell and Egli, 2010).

According to Baye et al. (2015), in order to achieve significant improvements in the Zn and Fe bioaccessibility of high tannin cereal grains, it is important for both the condensed tannin and phytic acid to be sufficiently reduced because these anti-nutrients are closely linked in the plant matrix.

This means that similar to the study by Gabaza et al. (2018), the anti-nutrients present during the manufacture of motoho were not adequately reduced to have had an effect on the bioaccessibility of Fe and Zn in this study.

Conclusion

Fermentation was shown to decrease the phytic acid and tannin content to a certain extent during the production of motoho. Future work on motoho could therefore entail refining the fermentation process in order to promote the growth of enzyme producing microorganisms, capable of adequately degrading the phytic acid to levels which may impact the bioavailability of Fe and Zn.

Some studies have shown that African cereal products can be fortified with other foods which contain high levels of Fe and Zn. Vilakati, Taylor, MacIntyre and Kruger (2016) demonstrated the increase in Fe and Fe bioaccessibility from 0.31 to 1.05mg/100g dm and 0.11 to 0.20 mg/100g dm respectively after a locally available legume called cowpea was added to sorghum. This could also be an avenue in which the bioavailability of Fe and Zn might be increased during the production of motoho.

Even though the uptake percentages were low for motoho, the motoho variant J showed the highest uptake for Mg, Fe and Ca, and slightly lower uptake for Zn and this variant could be selected for future research which may include pre-processing techniques such as decortication, soaking and germination during the fermentation process which could possibly result in dephytinization of motoho, thereby increasing mineral bioaccessability. Future studies on the motoho variety J could then also include upscaling and commercialization because there is a demand for fermented products in South Africa.

Author Contributions

Miss Sanchia Moodley conducted all the research for this article as well as writing the content for the article. She is the primary author.

Dr Angelique Botha and Miss Sophia Linsky developed the method for the ICP-OES, provided the reagents and equipment for testing the samples and analysed the samples on the ICP-OES.

Dr Lucia Steenkamp edited the article and has provided direction for the research methods. She is also a co-supervisor for this research.

Prof Vincent Gray edited the article and has provided guidance for the research methods. He is also the academic supervisor.

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Ethical statements

Ethical review: This study was approved by the University of the Witwatersrand Human Research Ethics Committee (Non-Medical).

Conflict of Interest: The authors declare that they do not have any conflict of interest.

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Table 1: The initial fermentation screening process. Fermentation of different motoho varieties using the different starter cultures and sorghum varieties with n=10 for each fermentation.

Sample	Sorghum variety	Starter culture
A (Control)	Regular commercial sorghum	Traditional
B	Regular commercial sorghum	*Lb. fermentum*
C		*Lb.plantarum*
D		*Lb. fermentum* + *Lb.plantarum*
E	SV2	*Lb. fermentum*
F		*Lb.plantarum*
G		*Lb. fermentum* + *Lb.plantarum*
H	Rakodzi	*Lb. fermentum*
I		*Lb.plantarum*
J		*Lb. fermentum* + *Lb.plantarum*
K	SC Smile	*Lb. fermentum*
L		*Lb.plantarum*
M		*Lb. fermentum* + *Lb.plantarum*
N	Macia	*Lb. fermentum*
O		*Lb.plantarum*
P		*Lb. fermentum* + *Lb.plantarum*

Table 2: Tannin, tannase, phytate and phytase results for motoho (A, J and M) and unfermented sorghum (regular commercial, Rakodzi and SC Smile) used to make the different motoho varieties. Values are means of ten replicates ± standard deviations. NM = not measured. U/ml = units of enzyme per ml of product and g/Kg-1 = grams per kilogram. CE G Kg-1 = catechin equivalent units (CE).

	A	J	M	Regular sorghum	Rakodzi	SC Smile
Tannin CE g Kg^{-1}	2.99.±9.80	28.72±42.95	37.55±23.27	51.38±21.57	162.06±90.56	255.16±105.22
Tannase U/ml	1.34±0.73	0.92±0.38	0.67±0.54	NM	NM	NM
Phytate g/Kg^{-1}	2572.01±575.70	1758.05±300.72	1896.05±471.35	2836±651.57	1776.67±689	1900.88±483.54
Phytase U/ml	0.26±0.06	0.35±0.06	0.44±0.05	NM	NM	NM

Table 3: The soluability percentages as well as calcium, magnesium, iron and zinc retention, transport and uptake from motoho varieties A, J and M by Caco-2 cells. Molar ratios for phytate: zinc, phytate: iron and calcium: phytate are also represented. Data represented are means and standard deviations with n=3.

	Motoho		
	A	**J**	**M**
Solubility %			
Calcium	1.7	4.83	2.67
Magnesium	1.58	1.52	1.25
Iron	0.49	0.67	0.65
Zinc	0.37	0.36	0.31
Calcium			
Retention(µg)	26.11 ±11.89	12.56±1.83	11.66±3.27
Retention %	5.59±1.07	9.25±1.35	4.75±1.33
Transport(µg)	14.25±2.75	23.53±4.60	18.45±5.18
Transport %	3.70±0.71	17.33±3.39	7.52±2.11
Uptake (µg)	40.36±9.46	36.09±4.03	30.11±1.91
Uptake %	10.47±2.45	26.58±2.97	12.27±0.78
Magnesium			
Retention(µg)	8.91±2.64	8.55±1.28	9.33±1.58
Retention %	0.64±0.19	0.59±0.09	0.53±0.09
Transport(µg)	7.50±1.64	9.98±3.40	6.05±4.04
Transport %	0.4±0.35	0.69±0.23	0.34±0.23
Uptake (µg)	16.41±1.14	18.52±2.70	15.38±2.92
Uptake %	1.17±0.08	1.28±0.19	0.87±0.17
Iron			
Retention(µg)	0.11±0.03	0.13±0.11	0.05±0.05
Retention %	0.23±0.06	0.39±0.33	0.11±0.16
Transport(µg)	0.03±0.02	0.04±0.01	0.02±0.02
Transport %	0.06±0.03	0.12±0.02	0.05±0.06
Uptake (µg)	0.13±0.03	0.17±0.11	0.07±0.03
Uptake %	0.29±0.07	0.51±0.33	0.20±0.08
Zinc			
Retention(µg)	0.07±0.02	0.07±0.02	0.06±0.02
Retention %	0.36±0.11	0.25±0.08	0.23±0.09
Transport(µg)	0.02±0.01	0.05±0.02	0.05±0.01
Transport %	0.09±0.04	0.18±0.09	0.21±0.05
Uptake (µg)	0.09±0.03	0.11±0.01	0.11±0.01
Uptake %	0.43±0.12	0.43±0.03	0.44±0.04
Molar ratios			
Phytate: zinc	11.8:1	6.5:1	11.10:1
Phytate: iron	4.76:1	4.36:1	6.89:1
Calcium: phytate	0.4:1	0.77:1	0.70:1

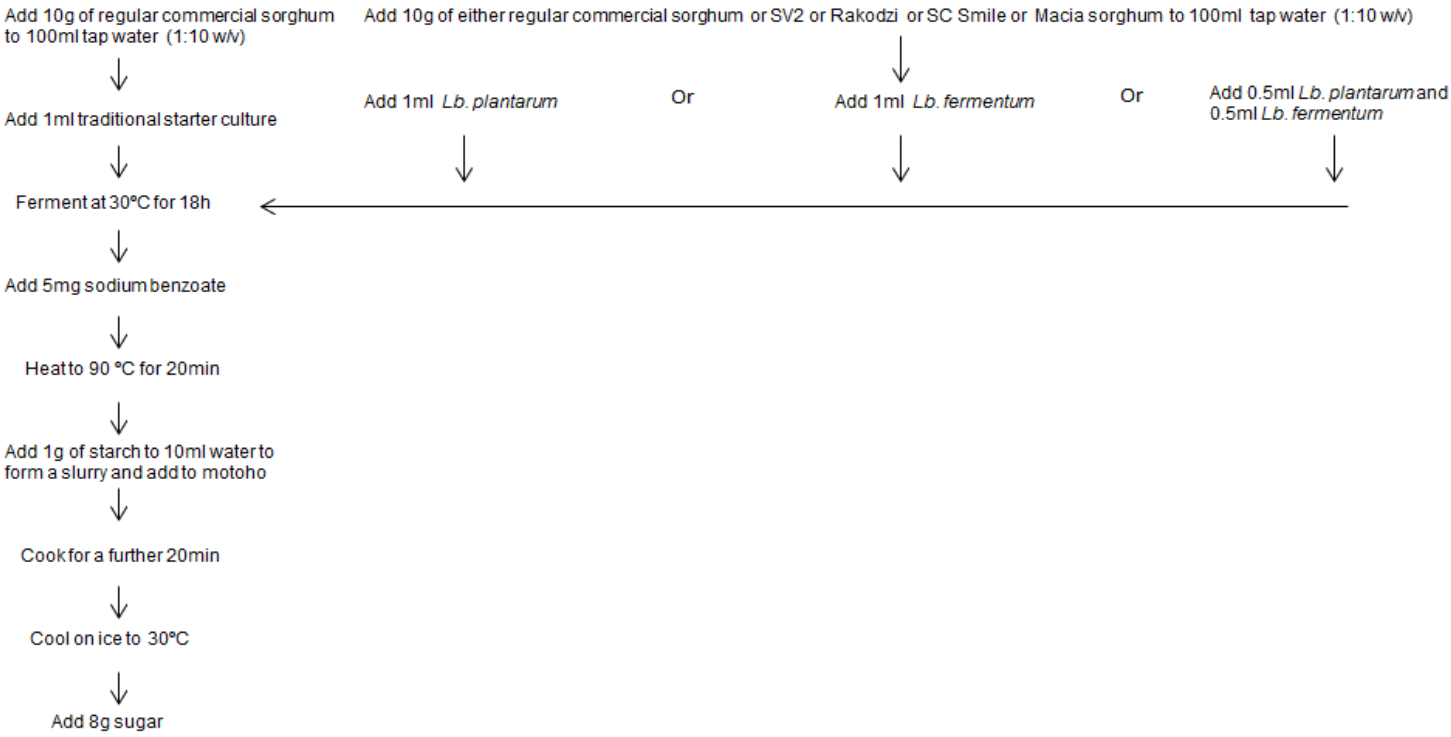


Figure 1 Flow chart outlining the production of motoho using traditional and defined starter cultures and commercially available and modified sorghum varieties. Ten replicates of each motoho were produced.

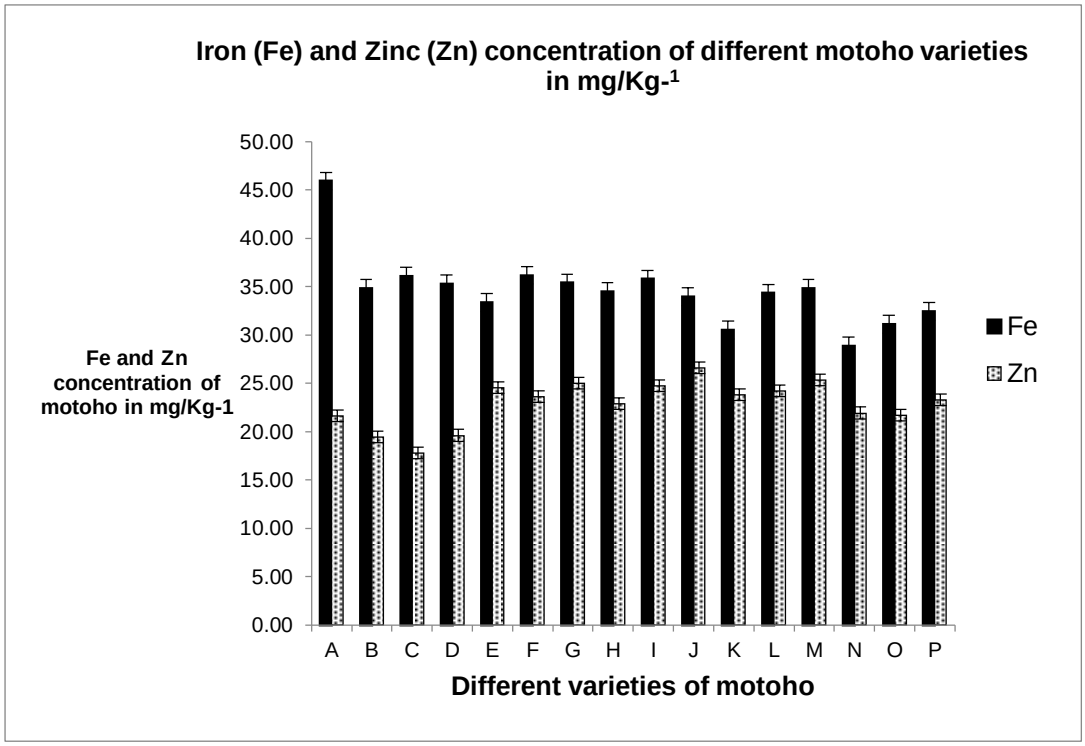


Figure 2: Iron and Zinc concentrations of the different varieties of motoho (A-P) in mg/Kg-^{1}. Values are means of ten replicates.

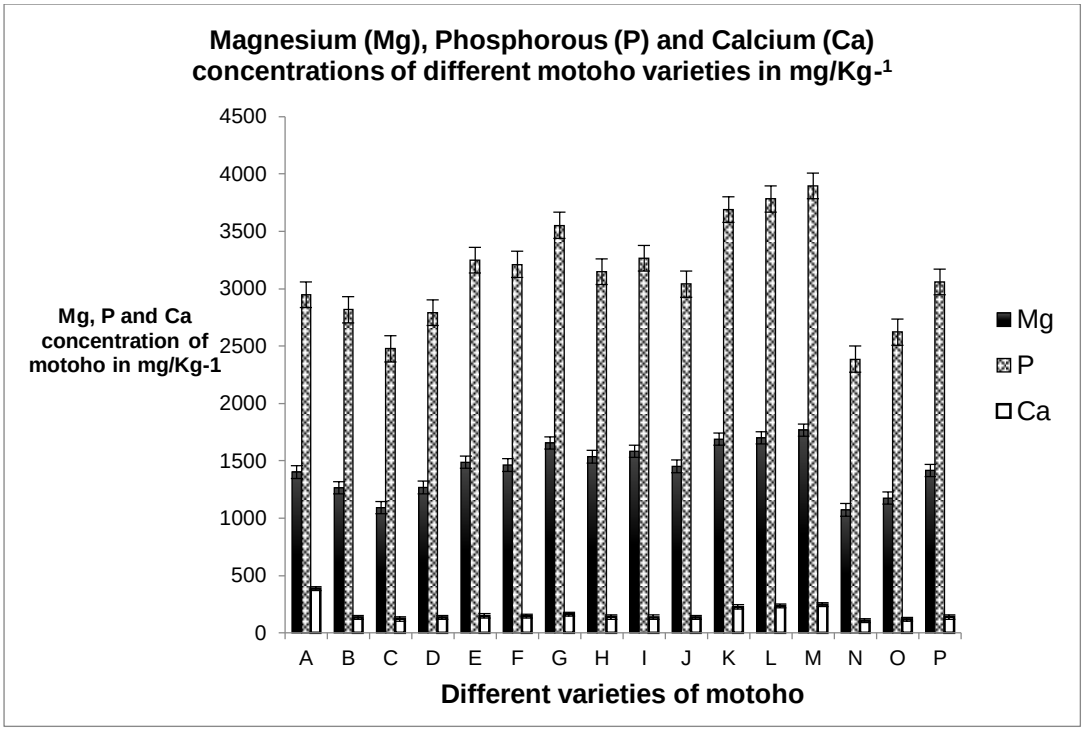


Figure 3: Magnesium, Phosphorous and Calcium concentrations of the different motoho varieties (A-P) in mg/Kg-^{1}. Values are means of ten replicates.

5 Conclusion

Traditionally fermented products are indigenous to certain regions and have been developed by indigenous people using age-old techniques as well as locally sourced raw materials. In many developing countries, traditional methods are passed down from generation to generation (Abegaz et al., 2002) and are garnering interest as a crucial part of food security strategies (van de Sande, 1997) and for commercial use. Motoho is a traditionally fermented sorghum beverage which is produced by the Basotho people of South Africa. Products made via spontaneous fermentation, which is an uncontrolled process and is subject to the microorganisms which originate from the sorghum, utensils or added as a traditional starter culture (Nout & Motarjemi, 1997) often have variable quality and sensory attributes (Chaves-López et al., 2014). Hence our first aim was to determine what the effect would be on the shelf-life and sensory acceptability of motoho if *Lb. fermentum* and *Lb. plantarum* strains were used as a defined starter culture during motoho fermentation. Three varieties of sorghum (regular, Rakodzi, SC Smile) and the traditional and modified methods of production were used to produce three different motoho varieties. The traditional motoho (the control) was produced using a traditional starter culture (*Tomoso*) and regular commercial sorghum whilst the defined starter culture and either Rakodzi or SC Smile sorghums were used to produce modified motoho.

The 5 day shelf-life study showed that the microbial counts obtained for motoho were temperature dependent, with higher microbial counts obtained from the motoho incubated at 37ºC than at 4ºC. However, at both temperatures, the microbial loads were lower than the recommended microbial load for beverages.

This implies that although it is recommended that motoho be consumed within 3 days, these studies have shown that motoho can be consumed even after 5 days. Overall, a sensory panel preferred the motoho produced from the Rakodzi sorghum.

It could be that the esters produced during the production of this motoho variety could have contributed to the preference in aroma and flavour. The next aim of the research was to determine what effect there would be on the micronutrient, polyphenolic and phytic acid contents of the different varieties of motoho. The Caco-2 uptake assay was then used in order to provide an estimate of the bioavailability of micronutrients from motoho. Initial screening was conducted using ICP-SFMS to determine which of the produced motoho varieties produced the highest Fe, Zn, Ca, Mg and P contents. The traditional motoho produced the highest Fe (45.91mg/Kg-1) and Ca contents (385.57mg/Kg-1) while the motoho produced with Rakodzi showed the highest Zn concentration (26.62mg/Kg-1). The motoho produced with SC Smile yielded the highest P and Mg contents of 3894.96 and 1768.20 mg/Kg-1 respectively. Tannase (0.67-1.64U/ml) and phytase (0.26-0.44U/ml) were produced during the motoho fermentations. However, sub-optimal growth conditions as well as deactivation of endogenous enzymes during the autoclaving of the sorghum could account for the low enzyme production during fermentation. The tannin content was reduced by 82.28-94.18% during motoho production while the phytate was minimally reduced (0.25-9.3%).

The uptake percentages for Fe and Zn during the Caco-2 assays were also much lower than what was previously found. Kruger et al. (2012) reported Fe uptakes of 0.76-0.98% and Zn uptakes of 1.21-1.89%. The studies on motoho showed Fe uptakes of 0.20-0.55% and Zn uptakes of 0.43-0.44%. Frontela et al. (2009) reported that phytate reduced the zinc bioavailability of three commercial infant cereals.

It is possible that the high phytate content in the motoho varieties could have decreased the mineral bioavailability since phytates form insoluble phytate-mineral complexes (Dvořáková, 1998).

Future research on the bioavailability of motoho could include methods to decrease the phytate content during production and in that way, mineral bioavailability may be improved. Overall, the motoho produced with the Rakodzi sorghum could be used for further research on motoho. It was the most preferred by a sensory panel in terms of flavour and also showed the highest uptake for Mg, Fe and Ca, and a slightly lower uptake for Zn.

5.2 References

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