Spontaneous fermentation of Munkoyo; a cereal-based beverage in Zambia

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Thesis

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CHAPTER – ONE

General introduction

Introduction

Fermentation is one of the oldest methods of preserving and processing food. Cereal-based traditional fermented products are the most frequently consumed fermented foods in rural areas in many African countries. This is driven by the fact that fermentation is an inexpensive technology that preserves food, improves its nutritional value and enhances sensorial properties. Fermentation relies on biological activity of microorganisms to transform raw material *(Kohajdova & Karovicova, 2007)*. Spontaneously fermented beverages play an important role in providing nutritious and safe foods that contribute to the livelihood of rural and peri-urban populations in Africa through enhanced food security and income generation by small–scale enterprises. Despite the wide-spread occurrence of these foods, information regarding traditional processing methods, microbiological characteristics, nutritional value and safety of the products is scarce *(Misihairabgwi & Cheikhyoussef, 2017)*. Different kinds of traditional fermented beverages follow different processing methods at household level across Africa. The most common fermented non-alcoholic cereal-based beverages are made from maize, sorghum and millet. They include Togwa in Tanzania, Mawe in Benin, Maheu in Zimbabwe and Munkoyo in Zambia *(Mugula et al., 2003, Hounhouigan, 1994, Gadaga et al., 1999, Zulu et al., 1997)*.

No starter cultures (i.e. defined mixes of microbes to start the fermentation process) have been developed for most of these beverages, hence the fermentation process is mostly spontaneous and thus driven by microorganisms from the environment, processing equipment or the normal microbiota of the substrate *(Motlhanka et al., 2018)*. Although these products have been consumed for many years as safe products, occasionally pathogens have been isolated that possibly survive and grow in some fermented foods *(Gadaga et al., 2004)*. This may be explained by the diversity of microorganisms present in and on the fermentable raw materials carrying possibly microbial pathogens or microorganisms producing toxic by-products such as mycotoxins, ethyl carbamate and biogenic amines *(Capozzi et al., 2017)*. Furthermore, most of these fermented beverages are processed under unhygienic conditions with inconsistent quality of raw materials, which reduces

the level of microbial safety and might cause a limited shelf-life. Thus, research into the processing technologies of these traditional beverages, including efforts to develop starter cultures, can be a milestone to ensure quality and safety.

Processing of Munkoyo

Zambia and the Democratic Republic of Congo are the two countries where a maize-based fermented beverage called Munkoyo is commonly produced and consumed *(Foma et al., 2012, Schoustra et al., 2013).* Munkoyo is originally the local name of the roots used in the processing of the beverage *(Foma et al., 2013).* The plant producing the root is known to be endemic to Zambia, the Democratic Republic of Congo, Zimbabwe, Angola, Tanzania, Malawi, Namibia, Botswana and Mozambique *(Foma et al., 2013).* It is also a native dominant shrub in the region between Lake Tanganyika and Lake Mweru *(Pauwels et al., 1992).* The genus name of this Munkoyo root and related species is *Rhynchosia (Zulu et al., 1994).*The processing of Munkoyo drink cannot be achieved without the use of enzymes, for which this root is the preferred source. Hence the beverage is named after the root. The principal raw material in the production of this beverage is maize meal, and the *Rhynchosia* root is thus used as an inoculum. The flow chart and the picture (Figure 1) show the general processing steps and the beverage after preparation.

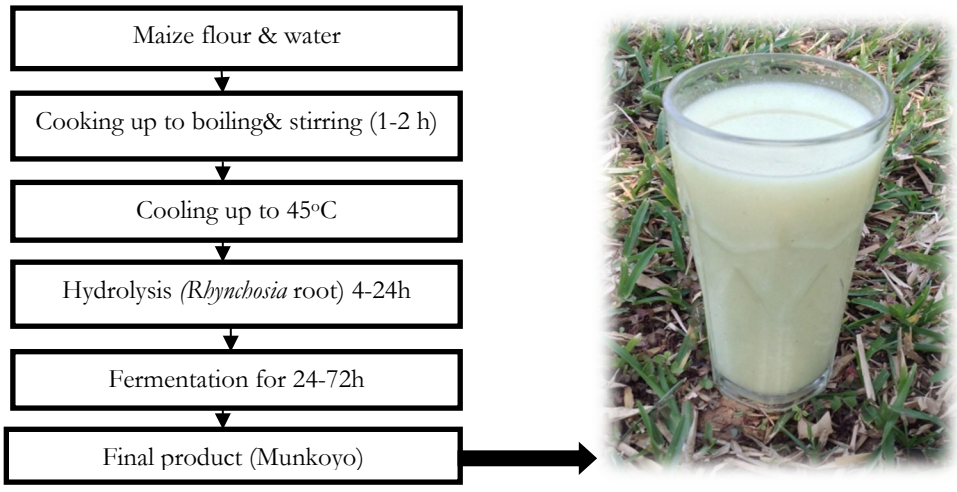

Figure 1. General processing steps for making Munkoyo. Maize flour (152 g) was mixed with 1L of water at 55°Cand cooked to gelatinize the starch, followed by hydrolysis of the starch through the enzymes derived from the roots and finally spontaneous fermentation by lactic acid bacteria.

Cooking (boiling) of the maize meal up to the point of gelatinization breaks down the intra and inter-molecular bonds of the long chains of starch into what can be degraded by the amylases into fermentable sugars. Gelatinization of starch modifies the α-(1-4)-glycosidic bond, causing the starch granules to absorb water and swell, thereby becoming more accessible to be degraded by enzymes. After cooling the gelatinized porridge to temperatures between 45°C and 55°C, *Rhynchosia* roots are added because they naturally contain a high amount of amylolytic enzymes, which break down starch into fermentable sugars *(Foma et al., 2013)*, and consequently reduce the viscosity of the gruel *(Mulkay et al., 1995)*. For example, pure amyloglucosidases have extensively been used in food processing industry such as baking, brewing, fruit juice and starch syrups because they are capable of hydrolysing amylose, amylopectin, maltose and malto-oligosaccharides into fermentable sugars, causing rapid loss of viscosity*(Shambe & Ejembi, 1987, Couto & Sanromán, 2006)*. Alpha amylase in particular are added to the dough of bread to degrade the starch in the flour into smaller dextrins which are subsequently fermented by yeast resulting in improved volume and texture of the product. The action of these α-amylase generates additional sugar in the dough which improves the taste, crust colour, toasting qualities of the bread and anti-stalling effect that increase the shelf life *(van der Maarel et al., 2002)*.

The use of the *Rhynchosia* roots in the production of Munkoyo makes it distinctly different from all other similar beverages across Africa. Most of the cereal-based beverages use malted sorghum or millet for the supply of amylases to degrade starch into fermentable sugars. Table 1 shows cereal-based beverages in Africa processed from maize, malt of sorghum or millet, important steps in processing and dominant bacterial strains. Previous studies have shown that the use of *Rhynchosia* roots allows degradation of starch, however, no study has documented the exact species within the *Rhynchosia* genus that is used *(Zulu et al., 1997, Foma et al., 2012)*, also see Chapter 2. The sugars produced during Munkoyo processing due to starch degrading enzyme activity are mostly maltose 80%, maltotriose 17% and glucose 3%. These sugars are subsequently converted by lactic acid

bacteria (LAB) into organic acids and characteristic aroma compounds in a process that is referred to as spontaneous fermentation *(Zulu et al., 1997)*. LAB are known to produce lactic acid from milk based products such as skim milk and whey or from starch-based products such as potatoes, cassava, wheat, rice or sorghum. It is specifically thefermentation of C5 and C6 sugars by lactic acid bacteria via either a homofermentative or heterofermentative metabolism, which leads to the characteristic sensorial attributes of the beverage *(Juturu & Chuan Wu, 2015)*.

Table 1: Traditional cereal-based fermented beverages in Africa; raw material, processing techniques and microbial species involved.

Beverage (Country)	Raw material	Processing steps	Microbial species	References
Mangisi (Zimbabwe)	millet/maize	cooking/malting/ filtration/fermentation	*Saccharomyces cerevisiae, Lactococcus lactis, Lactobacillus fermentum.*	*(Gadaga et al., 1999)*
Gowe (Benin)	maize/millet/ sorghum	cooking/malting/ fermentation	*Weissella confusa, L. fermentum, Pediococcus.*	*(Adinsi et al., 2014)*
Borde (Ethiopia)	barley/maize/ sorghum/teff/ wheat	malting/roasting/ fermentation	*Lactobacillus cellobiosus, Lactobacillus plantarum.*	*(Abegaz et al., 2004)*
Togwa (Tanzania)	maize/millet malt	cooking/malting/ fermentation	*Lactobacillus brevis, L.fermentum, S. cerevisiae.*	*(Mugula et al., 2003)*
Kenkey (Ghana)	maize/sorghum/ millet	steeping/grinding/ fermentation/ boiling	*Lactobacillus reuteri, L.fermentum, Penicilium, Fusarium.*	*(Annan et al., 2003)*
Kwete (Uganda)	maize/malted millet	germination/cooking/ fermentation	*W. confusus, L.plantarum.*	*(Namugumya & Muyanja, 2009)*
Maheu (S.Africa)	sorghum/maize	cooking,/malting/ fermentation	*L. lactis, L. brevis, Corynebacterium.*	*(Mugochi et al., 2001)*
Ogi (Nigeria)	maize/sorghum /millet	wet milling/sieving/ sedimentation	*Acetobacter, S.cerevisiae, Candida.*	*(Kosisochukwu et al., 2018)*
Oshikundu (Nambia)	sorghum/millet	backslopping/ fermentation/	*L.plantarum, Lfermentum, Lactobacillus curvatus.*	*(Misihairabgwi & Cheikhyoussef, 2017)*
Uji (Kenya)	maize/millet	germination/frying/ fermentation/filtration	*L.plantarum, Leuconostoc mesenteriodes,*	*(Waters et al., 2015)*

Spontaneous fermentation by lactic acid bacteria

Lactic acid bacteria (LAB) are the most important bacteria used in many food fermentation processes, including Munkoyo processing. LAB constitute a genetically and ecologically diverse group of non-motile microaerophilic Gram positive bacteria, including several genera of *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Oenococcus*, *Lactococcus*, *Streptococcus* and *Weissella* *(Hatti-Kaul et al., 2018).* Previous work has shown that during Munkoyo production the microbial communities are dominated by species of the genera *Weissella* and *Lactobacillus* *(Schoustra et al., 2013).* LAB ferment reduced sugars into lactic acid, vital for lowering the pH and the production of desirable sensorial attributes, In addition, certain LAB produce specific flavour compounds like diacetyl, acetoin, acetaldehyde and or acetic acid; some of which contribute to a typical yoghurt or butter flavour (*van Kranenburg et al., 2002).* Although LAB have an incredible value in food processing, their role in spontaneous fermentation of cereal-based beverages is still not properly clarified in scientific literature, which poses a challenge in targeting specific sensorial characteristics. Sensorial characteristics of fermented products dominated by LAB may not always be regarded as desirable by all consumers, and thus represent an important hurdle for their acceptance *(Nsogning Dongmo et al., 2016)*. Furthermore, spontaneous fermentation increases the chance of outgrowth pathogenic bacteria naturally present at low abundances in or on the raw material used for fermentation.

From spontaneous fermentation to starter culture development

Spontaneous fermentation is an ancient and most popular biotechnology used by humankind dating back 10,000 years from Neolithic period *(Motlhanka et al., 2018).* Its exploitation was mostly in dairy products, baking, wine making and brewing using wild microorganisms that drove fermentation but also significantly influenced flavor and quality of finished fermented products *(Morrison-Whittle & Goddard, 2018).* The disadvantage of using wild microorganisms in fermentation has been unpredictable quality and off-flavours in the product. In modern times, starter cultures

have been developed for many applications with the intention of making use of specific microbial properties to accelerate and control the fermentation process *(Leroy & De Vuyst, 2004, Hammes, 1990)*. LAB have been used in the development of such starter cultures because they have a critical role in fermentation processes with a long history of application and production of fermented foods and beverages. They ensure not only increased shelf life and microbiological safety of foods but also make some foods better digestible *(Caplice & Fitzgerald, 1999)*. Most single or multiple strain LAB based starter cultures are known to produce exopolysaccharides, organic acids, aromatic compounds or bacteriocins, which are released into the food matrix thereby giving improved texture, aroma, flavour, health benefits and a longer shelf life *(Leroy & De Vuyst, 2004)*. Hence, the selection of LAB for a specific starter culture needs sound knowledge of the ecological factors linked with the fermentable raw material (i.e. the substrate), an understanding of the role of microorganisms in the formation of aroma compounds, texture and the genetics of LAB involved in fermentation *(Buckenhüskes, 1993)*.

Spontaneously fermented cereal-based beverages in Africa contain LAB such as *Lactobacillus*, *Lactococcus*, *Streptococcus* and *Leuconostoc* *(Chelule et al., 2010)*.The fact that most commercially formulated starter cultures contain similar LAB as found in spontaneously fermented beverages*(Vázquez-Velázquez et al., 2018)*, paves the way towards the design of a specific starter culture for fermented cereal-based beverages. The use of such starters is expected to result in a high degree of control of the fermentation process and standardization of the end product.

Motivation for the study

Maize cereal is the most cultivated crop in Southern Africa because it is a staple crop. Fermented maize-based foods are increasingly becoming important because they are consumed by everyone and can be produced into an energy drink mostly consumed at many social gatherings. Recent studies show that fermented cereal-based beverages are ideal for lactose intolerant people, boost immune system, reduce occurrence of diarrheal disease by bacteria and malnutrition *(Chelule et al.,*

2015). Further, traditional fermentation of maheu, a cereal-based beverage in South Africa is known to increase protein digestibility and detoxification of mycotoxins contained on maize in Southern Africa *(Chelule et al., 2010)*. For that reason fermented foods are used as weaning foods for infants to curb the problem of under nourishment and diarrheal disease in children.

Munkoyo is equally gaining importance as a beverage to address food security in rural areas where food shortages may be prevalent because of low cost of producing it and readily availability of maize as a staple crop. This is already evident in Namibia, where Oshikundu, a similar cereal-based beverage like Munkoyo, is carried to school by children as an energy drink. Nursing mothers and the elderly are encouraged to consume Oshikundu as an energy replenishing beverage *(Embashu et al., 2013)*. Similarly, the optimization and control of processing cereals into Munkoyo has a potential to improve the socio-economic status of rural communities through employment creation, increasing family income, providing a non-seasonal supply of safe and nutritious beverage as observed for other non-cereal based beverages across sub-Saharan Africa *(Motlhanka et al., 2018)*. Therefore, Munkoyo can be important in alleviating hunger, improving nutrition and health if regularly included in daily diets but also contribute to the country's economy if it gets into processing at an industrial scale.

Fermented foods are an important part of the diet in many cultures that primarily have been used to preserve food, enhance shelf life and improve flavour. The beneficial effects of fermented foods on health is due to excellent functional and nutritional properties as a result of fermenting microorganisms and bioactive compounds released *(Achi & Asamudo, 2018)*. Until recently, not much research effort has gone into improving the production process, sensorial attributes and nutritional aspects of Munkoyo like what has been done for other cereal-based beverages across Africa like (Ogi) in Nigeria, (Mawe) in Benin, (Togwa) in Tanzania and (Maheu) in South Africa *(Kosisochukwu et al., 2018, Hounhouigan, 1994, Mugula et al., 2003, Mugochi et al., 2001)*. Some studies have been done on physico-chemical properties and identification of bacteria involved in the

production of Munkoyo *(Simwamba & Elahi, 1986, Schoustra et al., 2013, Foma et al., 2012)*. However, the link between processing methods, microbial composition, aroma compounds produced and nutrition benefits as a result of fermentation process have not been reported at all.

This study therefore aims at documenting the traditional processing methods of making Munkoyo and understanding the link between the microbiological changes that take place during spontaneous fermentation and the aroma profiles produced as a result. This includes identification of microorganisms involved in the spontaneous fermentation of Munkoyo, followed by the selection of microorganisms for possible starter culture development.

To achieve this goal, the specific objectives of the thesis were to study and document:

- the traditional processing methods of producing Munkoyo
- variations in processing methods and how these affect the composition of the microbial community responsible for the spontaneous fermentation
- the link between selected lactic acid bacteria and the aroma profiles produced during spontaneous fermentation
- the role of the *Rhynchosia* root in spontaneous fermentation

The overall research approach to address these objectives is shown below (Figure 2).

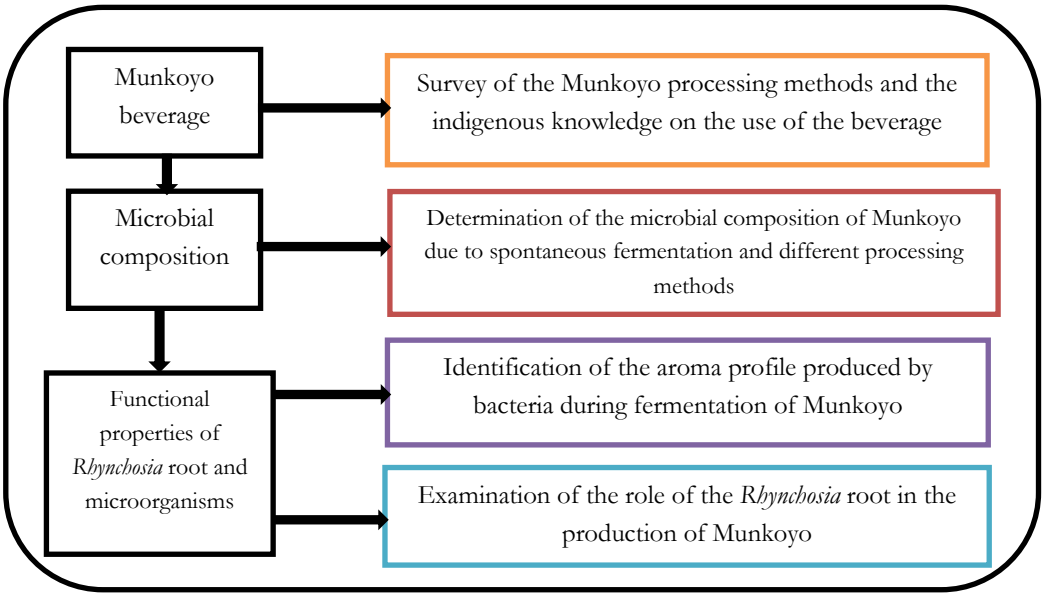

Figure 2. Schematic overview of the objectives of the study of spontaneous fermentation of Munkoyo

Outline of the thesis

The general introduction of this thesis gives an overview of the characteristics of Munkoyo, the general processing procedure of making the beverage, the most common bacteria involved in fermentation, aroma compounds produced and the role of the *Rhynchosia* root in the production of Munkoyo. **Chapter 2** presents the results of a survey on how, where and when the beverage is consumed and by whom. It further identifies the microbial communities and aroma compounds produced in the beverage. **Chapter 3** describes a study on how different processing methods in different agro-ecological zones of Zambia affect the composition of microbial communities. **Chapter 4** determines the aroma compounds produced from selected pure strains of most common bacteria and mixtures thereof in cereal-based beverages, and then compares these with the aroma compounds found in traditional (i.e. spontaneously fermented) Munkoyo.

Chapter 5 examines the role of the *Rhynchosia* root in the production of Munkoyo. **Chapter 6** concludes the thesis with a general discussion, findings of the research and recommendations for further studies.

CHAPTER - TWO

Fermented cereal-based Munkoyo beverage: processing practices, microbial diversity and aroma compounds

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Abstract

Fermented cereal-based foods play a crucial role in attaining food and nutrition security for resource-poor populations in sub-Saharan Africa. These products are widely produced by spontaneous fermentation using cereal grains as raw material. They have a unique taste and flavour, rich source of energy and their non-alcoholic nature makes them ideal for consumption by the entire population, including children. Lactic acid bacteria dominate the fermentation process and lead to a low pH of around 4, which suppresses the growth of pathogenic bacteria, thereby increasing the shelf-life and safety of the food. Knowledge about processing practices, consumption patterns and bacterial communities is essential to regulate processing and design appropriate mixes of micro-organisms to produce starter cultures for commercial production of standard-quality fermented foods that meet desired quality characteristics. In four regions of Zambia, we surveyed processing practices and consumption patterns of a spontaneously fermented cereal-based beverage called Munkoyo, commonly produced in Zambia and the Democratic Republic of Congo. Variations in processing practices exist in cooking time of the unfermented maize porridge and time allowed for fermentation. Consumption is mainly at household level and the product is considered as an energy drink. Characterization of the bacterial communities of over 90 samples with 16S amplicon sequencing on DNA extracted from the entire bacterial community revealed six dominant families, namely Streptococcaceae, Leuconostocaceae, Enterobacteriaceae, Lactabacillales, Bacillaceae and Aeromonadaceae, with a Shannon index of up to 1.18 and an effective number of 3.44 bacterial species. Bacterial communities that underlie the fermentation in Munkoyo differ in their composition for the different regions using common processing steps, suggesting that different combinations of bacteria can be used to achieve successful Munkoyo fermentation. Analysis of aroma profiles in 15 different samples from two different Provinces showed that aldehydes, esters, organic acids, alkanes, alkenes and alcohols dominated.

Introduction

The production of fermented beverages is one way to utilize cereals for human consumption. Fermentation is known to restrict the proliferation of bacterial pathogens, resulting in an increased shelf-life and microbial safety of these products. The main mechanism for this functionality is that of lowering the pH to values below 4 by the production of lactic acid bacteria *(Nout & Motarjemi, 1997).* Moreover, fermentation leads to a generally perceived improvement in texture, taste and aroma of the final product due to the development of a complex blend of texture and flavour compounds *(Nout & Motarjemi, 1997, Oyewole, 1997)*. In addition, advances in scientific knowledge have taught us other benefits of the activities of micro-organisms in food preparation, such as the development of health supporting properties due to vitamin production and anti-diarrheal attributes *(Kort & Sybesma, 2012).*

Around the world, many traditional cereal-based fermented beverages exist, both alcoholic and non-alcoholic *(Mugula et al., 2003, Soro-Yao et al., 2014, Nout, 2009).* These beverages are frequently consumed because they are inexpensive to prepare and do not require refrigeration or pre-heating prior to consumption *(Nout, 2009).* As a result of the appetizing taste and flavour, adults and, in the case of the non-alcoholic products, children alike consume cereal-based beverages, for instance at major ceremonies such as weddings or funerals. The fact that the preparation of these beverages is based on spontaneous fermentation entails that the process is not controlled regardless of the vessel used. This leads to a diverse microbial flora from the local environment, besides variations in the production process. The way in which microbial communities develop during spontaneous fermentation, depends on the food ingredients and the surrounding environment in addition to the interaction of the micro-organisms themselves *(Mugula et al., 2003, Hounhouigan et al., 1993, Nout, 2009).* In non-alcoholic products, lactic acid bacteria dominate, of which the predominant lactic acid bacteria (LAB) in most cereal-based fermented beverages include *Lactobacillus, Lactococcus, Leuconostoc* and *Pediococcus.* The *Lactobacillus* species include *L. fermentum*, *L. plantarum* and *L. Delbrueckii (Nout, 2009, Oyewole, 1997).*

Sensorial properties of fermented foods are largely determined by micro-organisms, which makes identification of the microbial communities and their diversity crucial to understand how flavour and taste of these products come about. Aroma compounds are a key component of these sensorial properties *(Mugula et al., 2003, Oyewole, 1997)*. These aroma compounds consist of many volatile and non-volatile compounds, which possess diverse chemical and physicochemical properties. They include alcohols, aldehydes, esters, di-carbonyls, short to medium-chain free fatty acids, methyl ketones, lactones, phenolic and sulphur compounds. The non-volatile compounds largely contribute to the taste, whilst volatile compounds influence both the taste and flavour *(Longo & Sanroman, 2006, Blandino et al., 2003, Vieira-Dalode et al., 2016)*. Especially the presence of diacetyl, acetic acid and butyric acid makes fermented beverages appetizing to a large group of consumers *(Blandino et al., 2003)*.

Most African traditional fermented beverages are widely consumed and embedded in the local culture *(Oyewole, 1997)*. They are mostly produced at household level. Details on processing practices and/or fermenting microbes are largely unreported, although some have been documented *(Mugula et al., 2003, Hounhouigan et al., 1993, Sacca et al., 2012, Nguz et al., 2004, Moonga et al., 2019)*. As a result, little is known about the composition and diversity of micro-organisms that drive the fermentation process. Lack of this information impedes formal and up scaled development of these products, resulting in, amongst others, a rapidly increasing number of mostly urban consumers who do not have access to the traditional products that are part of their cultural heritage. Rapid urbanisation leads to an increasing urgency to take the production of traditional and culturally embedded foods to the next level.

This is also true for Munkoyo, a traditional cereal-based fermented beverage from Zambia and the Democratic Republic of Congo *(Schoustra et al., 2013, Foma et al., 2012)*. Previous work has described the general features of processing and has found that various groups of LAB such as *Lactobacillus plantarum*, *Weissella confusa*, *Lactococcus lactis* and *Enterococcus italicus* are present in the final product

(Schoustra et al., 2013). These microbes likely drive the fermentation and hence determine the type of final product and its properties. The first aim of this paper is to survey the current state-of-the-art in the production of Munkoyo from different regions in Zambia and to establish the main reasons for consumption. The second aim is to measure physicochemical properties and to profile Munkoyo samples for their bacterial community composition, as linked to sampling location, variations in processing practices and consumption preferences. The third aim is to identify the aroma compounds of the beverage that provide the unique sensory attributes to Munkoyo. Knowledge about microbial communities and their processing practices is essential to be able to standardize production of the beverage and advance the understanding of the factors that drive the species composition of fermenting microbes. A standardized production process will benefit from the use of defined mixes of micro-organisms (i.e. starter cultures), which facilitate controlled production of fermented foods to optimally meet the sensory quality characteristics desired by consumers *(Soro-Yao et al., 2014).*

Materials and methods

Questionnaires on traditional processing practices

A questionnaire was designed to assess prevailing processing procedures as well as consumption preferences and patterns of Munkoyo. Questionnaires were administered to consumers and processors of Munkoyo at 4 locations in Zambia: Mumbwa, Chibombo, Lusaka and Chongwe. All locations are in the same middle rainfall agro-ecological zone (II) (Fig 1). Respondents were selected by the 'snowballing' method, defined as a non probability sampling technique that cannot be statistically signified *(Katz, 2006).* Camp Agricultural Extension officers (CEOs) selected participants based on their willingness to participate in the research. A total of 172 participants aged between 19 and 60 years, comprising 62 males and 110 females, filled in the questionnaires, which included questions on raw materials, treatment steps, timing of these steps, type of fermentation vessel and the addition of supplementary materials to facilitate fermentation, such as roots from a special plant called *Rhynchosia.* Questions on consumption patterns and/or use of

Munkoyo focussed on consumed quantities, who mostly consumed it, at what occasions and main motivation for consumption.

Figure 1. Sampling locations. Middle rainfall agro-ecological zone where questionnaires were administered and samples of Munkoyo collected to assess the prevailing processing procedures and consumption pattern.

Physical properties and analysis of the microbial species

Munkoyo samples were collected from more than 50% of the questionnaire respondents randomly selected from the four locations of the research, amounting to 96 samples for analysis of pH, titratable acidity and bacterial species composition. pH was recorded using a portable pH meter (*Mettler Toledo AG*). Titratable acidity was determined by titrating 10 cm^3 of the sample against sodium hydroxide with phenolphthalein as an indicator. Bacterial species composition was determined based on 16S DNA amplicon sequencing. For this, all DNA of the bacteria was extracted from each sample, following the research of Schoustra et al 2013 *(Schoustra et al., 2013)*,as follows. One ml of Munkoyo sample was spun down at high speed, after which the supernatant was discarded. Next, 500 µl TESL, 10 µl mutanolysin solution and 100 µl lysozyme solution were added to the pellet and incubated at 37°C for 60 min with slight shaking. Then 500 µl GES reagent

was added, followed by cooling on ice for 5 min. Subsequently 250 µl of cold ammonium acetate solution was mixed gently, followed by keeping the mixture on ice for 10 min before spinning and collecting the supernatant. The supernatant was purified with chloroform-2-pentanol by mixing 1:1, spinning down at 12,000 rpm and collecting the supernatant. DNA was precipitated by adding 0.1 volume of 3 M sodium acetate followed by 2.5 volumes of 100% ethanol, and storage at -20°C overnight. Next the mixture was spun for 20 min at 12,000 rpm at 4°C and the supernatant removed. Finally, the DNA pellet was washed out by adding 1 ml of cold 70% ethanol and spinning for 10 min at 12,000 rpm at 4°C. After removal of the supernatant, the DNA pellet was air dried for 10 min at room temperature, dissolved in 10 mM Tris pH 7.5.The purified DNA was sent to LGC Genomics in Berlin, who performed 16S amplicon sequencing. They first performed a PCR using about 1-10 ng of DNA extract (total volume 1 µl), 15 pmol of each forward primer (341F; CCTACGGGNGGCWGCAG) and reverse primer (785R; GACTACHVGGGTATCTAATCC) (in 20 µL volume of 1 x MyTaq buffer containing 1.5 units MyTaq DNA polymerase (Bioline) and 2 µl of BioStabII PCR Enhancer (Sigma)). For each sample, the forward and reverse primers contained a unique 10-nt (company specific) barcode sequence. PCRs were carried out for 30 cycles, using the following parameters: 2 min 96°C pre-denaturation; 96°C for 15 s, 50°C for 30 s, 70°C for 90 s. About 20 ng amplicon DNA of each sample were pooled for up to 48 samples carrying different barcodes. If needed, PCRs showing low yields were further amplified for 5 cycles. The amplicon pools were purified with one volume AMPure XP beads (Agencourt) to remove primer dimer and other small mispriming products, followed by an additional purification on mini elute columns (Qiagen). About 100 ng of each purified amplicon pool DNA was used to construct Illumina libraries using the Ovation Rapid DR Multiplex System 1-96 (NuGEN). Illumina libraries were pooled and size selected by preparative gel electrophoresis. Sequencing was done on an Illumina MiSeq using V3 Chemistry (Illumina). Raw data were obtained from LGC Genomics and served as input for data analysis, for which we developed a pipeline. Bioinformatics and data analysis generated by DNA sequencing went through a rigorous quality system, which involved

identification and removal of sequences containing more than one ambiguous base (N) and evaluation of the presence and complementarity of primer and barcode sequences. For further data processing and statistics the QIIME pipeline *(Caporaso et al., 2010)*, modified from Bik et al *(Bik et al., 2016)* was used. Paired-end reads were joined using join_paired_ends.py (with minimum overlap 10 basepairs) after which sequences were trimmed and filtered using cutadapt (v1.11 -q 20, -m 400, Martin 2011) using the known primer sequences CCTACGGGNGGCWGCAG and GACTACHVGGGTATCTAAKCC to trimmed both sides of the sequence. These trimmed sequences were then checked for chimera's, using uchime (v4.2.20, gold database), *(Edgar et al., 2011)* with sequences with a lower chimera score than 0.28 were retained. After these trimming and filtering steps sequences were clustered into operational taxonomic units (OTUs) after quality check using pick_open_reference_otus.py (-s 0.1, -enable_rev_strand_match TRUE, -align_seqs_min_length 75, -pick_OTU_similarity 0.95). Taxonomy of the resulting OTUs was assigned to representative sequences using the Greengenes (v13.5) rRNA database. This algorithm gives a representative sequence for an OTU, which were used to perform a local blast using the gold database from uchime. The taxonomy from the top BLAST hit was used for further data processing.The anosim nonparametric test from the vegan package, use from the compare_categories.py wrapper in QIIME was used to test for significant differences in OTU tables between treatment factors (1000 permutations). Furthermore we used group_significance.py to perform a Chi-square testfor differences in abundance between individual OTUs between treatment levels.

Analysis of aroma compounds

Fifteen samples of Munkoyo, representative of all four surveyed locations, were selected for aroma analysis. Samples were defrosted and put in triplicate in 2 ml GC-MS vials, which were subsequently tightly closed to avoid loss of aroma compounds. Next the volatile compounds were extracted for 20 min at 60°C using a SPME fibre (Car/DVB/PDMS, Supelco). The compounds were desorbed

from the fibre for 2 min on a Stabilwax- DA-Crossbond-Carbowax-polyethylene-glycol column (30 m length, 0.25 mmID, 0.5 μm df). The gas chromatograph settings were: PTV Split-less mode (5 min) at 250°C. The carrier gas was helium with a constant flow of 1.5 ml/min. The GC oven temperature was set at 40°C for 2 min and later raised to 240°C (10°C/min) and kept at this temperature for 5 min. Mass spectral data was collected over a range of m/z 33-250 in full scan mode with 3.0030 scans/sec. GC-MS results were analysed using Chromeleon 7.2 software. The generated signal peaks were identified as aromatic products according to their elution time and relative area peaks. Using a library in Chromeleon, the names of the aroma compounds were identified. Area peaks, molecular weights and retention times were recorded.

Statistical analysis

The Statistical Package for Social Sciences (IBM SPSS statistic 23) and Microsoft Excel 2016 were used to analyse the responses from the questionnaires. Chi-square test generated the degrees of freedom (df) and the p-value determined the significant differences in the processing methods and consumption patterns.

Results

Processing practices and consumption patterns

This study systematically assessed the processing of Munkoyo using questionnaires of 172 consumers and producers at four locations in Zambia. Common steps were found to include soaking of maize meal in water, cooking of the mixture, cooling and temporary incubation with *Rhynchosia* roots as shown in Fig 2.

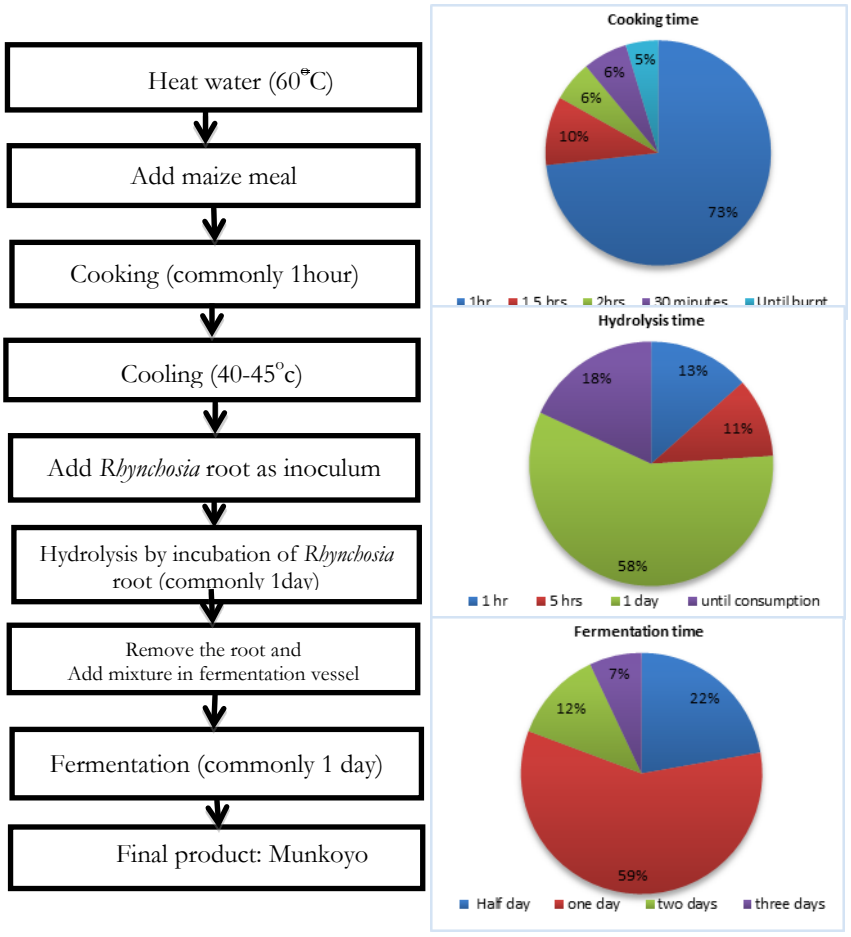

Figure 2. Flow diagram indicating processing steps of making Munkoyo and pie charts showing common variations in the duration of cooking the maize/water mixture, hydrolysis by the enzymes from *Rhynchosia* roots and fermentation in the fermentation vessels (buckets or calabashes).

The main variations in processing concern the duration of three critical steps: cooking of the initial maize porridge (cooking time), incubation with *Rhynchosia* roots (hydrolysis time) and fermentation by micro-organisms (fermentation time). The cooking time ranged from 30 min to several hours; most common duration was one hour. This cooking time is known to be adequate to gelatinize the starch for the action of enzymes supplied by *Rhynchosia* roots *(Zulu et al., 1997)*. Roots were beaten and stripped off prior to addition to the gelatinized porridge to increase the surface area of the roots, allowing the release of amylolytic enzymes into the water/maize mix to degrade the gelatinized starch into fermentable sugars. A temperature of around 45°C is optimal for hydrolysis of starch. Time allowed for incubation with roots varied between one hour and several days, most commonly and practically done overnight. *Rhynchosia* roots were not the only sources used to supply amylolytic enzymes; around 5% of surveyed producers used millet malt, cowpea roots and/or sweet

potato peels. Finally, the mixture with fermentable sugars was incubated in fermentation vessels for spontaneous fermentation for a period ranging from half a day to two days (most commonly one day) before consumption and ongoing for mostly three days until the beverage was consumed completely.

The most commonly used fermentation vessel was the plastic container as indicated in Fig 3, because it is easily available. However historically, mostly calabashes were used. Calabashes are still used on occasion, especially by producers aiming at sales, because previously used calabashes are known to quickly ferment the beverage as they harbour bacteria on the walls of the calabash. Earthen ware and metal buckets are no longer common as fermentation vessel.

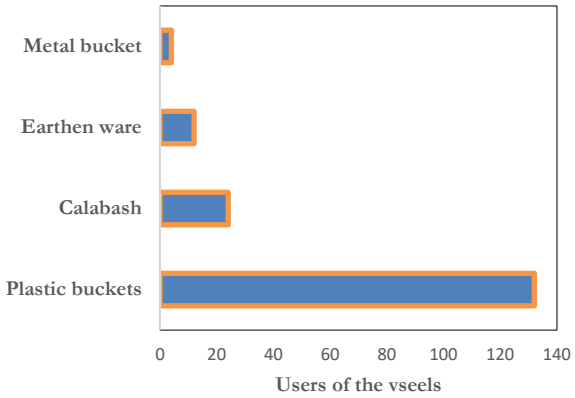

Figure 3. Frequency of the type of fermentation vessel used for the production of Munkoyo, as number of users among the respondents of the questionnaire. Plastic buckets, which are easily available and durable are commonly used vessels as compared to calabash, earthen ware or metal pot.

Fig 4 shows consumption patterns in different regions. The beverage is generally considered as an energy drink or snack, but is also consumed as a special drink at social gatherings such as weddings and funerals. However, people in formal employment with alternative options would only consume Munkoyo beverage at such social gatherings. The beverage is consumed by men and women of all ages, including children. They consume Munkoyo mainly when feeling hungry and at household level, which includes when working on the fields or during long distance travelling.

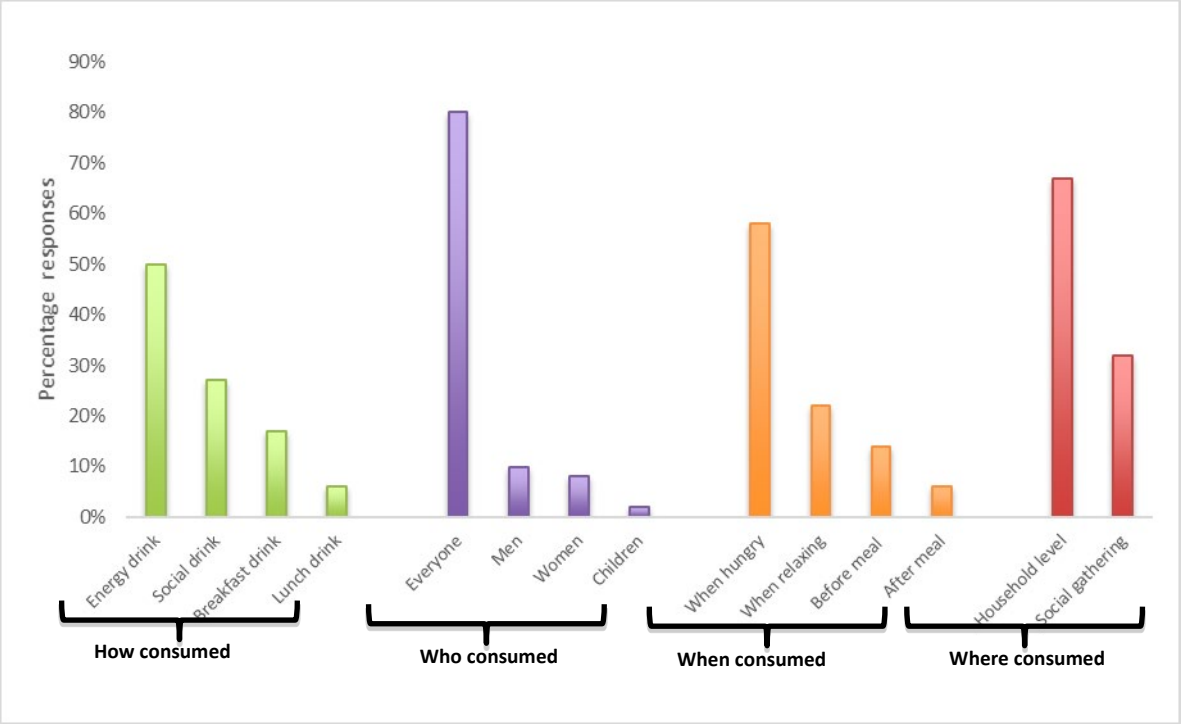

Figure 4. Consumption pattern of Munkoyo in the four surveyed locations outlining how the beverage was consumed, who consumed it, and when and where it was consumed.

The observed variations in processing in relation to the time of cooking, hydrolysis, fermentation and variations in consumption patterns per sampling location or province were statistically analysed as shown in Table 1.

Table 1. Chi-square test indicating the relationship between processing parameters and consumption patterns of Munkoyo. Processing parameters and consumption patterns with P-values in the same row with different letters are significantly different (p-value<0.05; α after Bonferroni correction to correct for multiple testing is 0.05/14=0.0035)

Contrast	Statistics	Cooking time	Fermentation time	Hydrolysis time	How Consumed	Who Consumed	Where Consumed	When Consumed
Province	Chi-square	1,737	4,667	13,542	10,139	5,838	0.107	19,879
	df	3	2	2	3	3	1	3
	p-value	0.629[b]	0.097[b]	0.001[a]	0.067[b]	0.120[b]	0.743[b]	0.000[a]
Location	Chi-square	2,717	5,343	7,222	6,757	6,912	0.527	28,468
	df	3	2	2	2	3	1	3
	p-value	0.437[b]	0.069[b]	0.027[a]	0.080[b]	0.075[b]	0.468[b]	0.000[a]

We found no significant differences between sampling locations in terms of cooking time, fermentation time, how the beverage is consumed, who consumed it and where it is consumed.

However, there was a significant difference in different provinces and locations with respect to the hydrolysis time and when the beverage was consumed. The difference in hydrolysis time could be as a result of using another type of inoculum other than *Rhynchosia* roots (i.e. millet malt, cowpea roots, sweet potato peels) for hydrolysis. The difference in when the beverage is consumed could be due to a difference in the social status of the consumers between locations and provinces. High social status groups tend to consume Munkoyo when relaxing whilst the majority low status groups consume Munkoyo as an energy giving food when they are hungry.

3.2 pH and titratable acidity

The pH of Munkoyo samples from different locations ranged between 3 and 4 and titratable acidity between 0.2 and 1% (Fig 5*)*. For both parameters, the values are statistically different per sampling location (ANOVA: pH, $F_{3,44}$= 7.31, P = 0.00044; TTA, $F_{3,44}$ = 12.61, P < 0.0001).

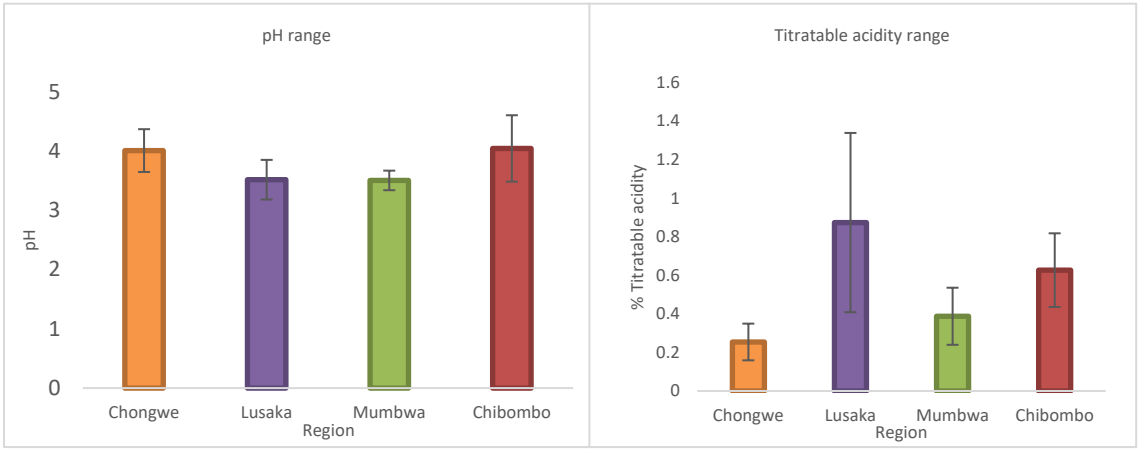

Figure 5. pH values and titratable acidity (TTA) of Munkoyo samples from Chongwe, Mumbwa, Chibombo and Lusaka. Error bars show standard deviation. The values for pH and for TTA are significantly different per sampling location.

These pH and titratable acidity ranges have been recorded for most cereal-based beverages in Africa, such as Mangisi of Zimbabwe with a pH of 3.98, titratable acidity of 0.67% and lactic acid concentration of 4.10 g/L (Zvauya et al., 1997).

Microbial composition and diversity

The bacterial composition of 96 samples was assessed based on 16S DNA amplicon sequencing of the V3-V4 region of the gene coding for the 16S RNA ribosomal subunit. This procedure yields over 10,000 reads per sample of around 350 base pairs each. Using bioinformatics analysis, we counted the number of unique sequence types (i.e. operational taxonomic units or OTUs) within each sample. We blasted the DNA sequence of each obtained read to a database to determine which species this unique sequence type had the highest similarity. Different unique types (OTUs) that blasted to the same species family were taken together when estimating diversity (the number of species present and their relative distribution). The analysis revealed over 42 different bacterial species within the microbial communities of the samples. In general, each sample contained up to eight dominant types, which each occupied at least 2% of the total population (Figure 6).

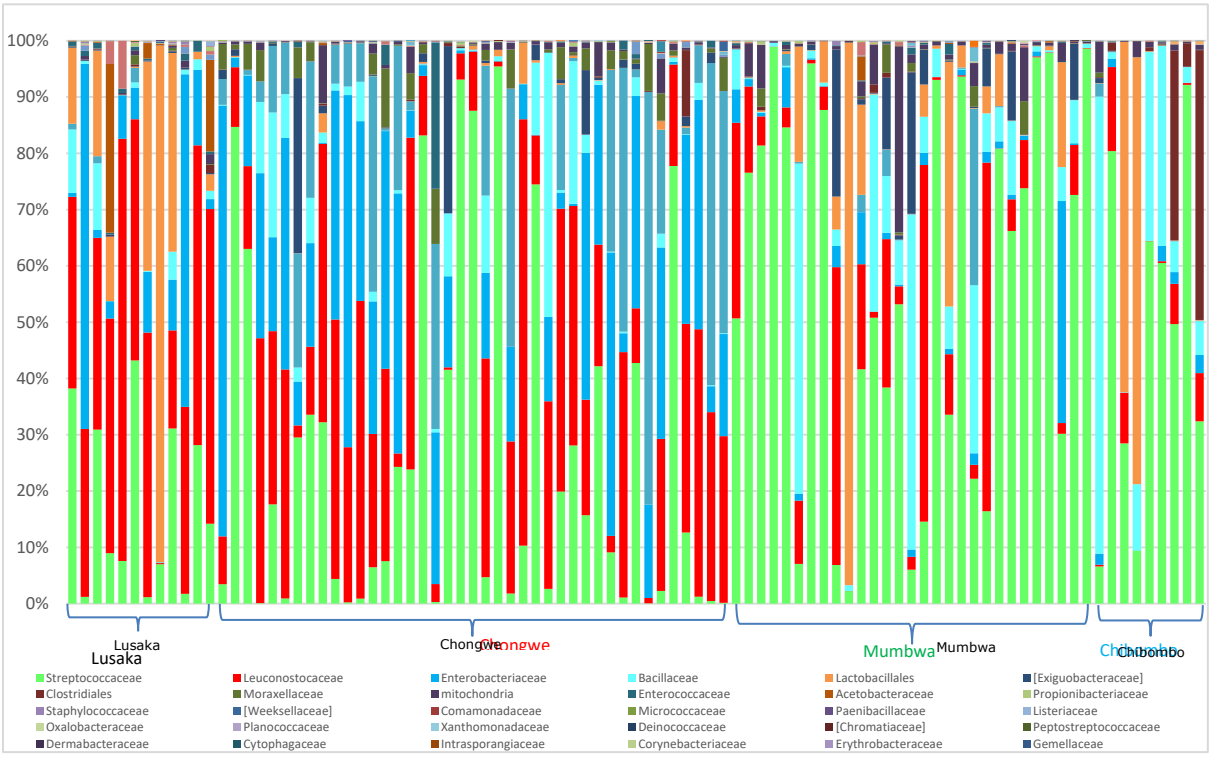

Figure 6. Bacterial composition in Munkoyo from different regions. Bars show relative abundance. Different colors show different families. Within each family multiple OTUs as well multiple species exit. Streptococcaceae were present in all the samples. Leuconostocaceae and Enterobacteriaceae dominated in samples from Lusaka province (Lusaka and Chongwe) whilst Lactobacillales and Bacillaceae dominated in samples from Central province (Mumbwa and Chibombo).

In several cases, species were represented by more than one type (i.e. OTU blasting to the same species), so at the level of unique types, the diversity was higher. Diversity measures include Shannon index (H), evenness and effective number of species (ENS). These measures were similar between sampling locations, highlighting that only slight variations in species distribution existed between the sampling locations. These differences between sampling locations were not statistically significant as observed in Table 2. (One-Way ANOVAs; Shannon $F_{3,69} = 1.751$, $P = 0.147$; Evenness $F_{3,70} = 0.724$, $P = 0.541$; ENS $F_{3,70} = 1.846$, $P = 0.147$). Additionally, the Shannon index number is of importance in projecting total biodiversity when it is used to estimate the effective number of species *(Morris et al., 2014)*. This estimated Effective Number of Species (ENS) is calculated by getting the exponential of the Shannon index, mathematically expressed as: $ENS = e^{H}$ *(Ugland et al., 2003)*.

Table 2. Average Shannon index (H), evenness and effective number of species (ENS) of all samples per sampling location. Numbers between brackets show standard deviation.

Location	Shannon index (H)	Evenness	ENS
Chibombo	0.86 (0.30)	0.27 (0.23)	2.47 (0.71)
Lusaka	1.18 (0.33)	0.28 (0.080)	3.44 (0.98)
Chongwe	1.14 (0.45)	0.43 (0.64)	3.11 (1.43)
Mumbwa	0.87 (0.52)	0.21 (0.12)	2.57 (1.38)

Thus, ENS estimates real biodiversity. In this case an ENS of 3.44 and 3.11 for Lusaka and Chongwe, respectively, indicates more diversity compared to Chibombo and Mumbwa with an ENS of 2.47 and 2.57, respectively. A Venn diagram (Fig 7) indicates at least 21 common species between the two provinces, with Lusaka province having more uncommon species across the regions (24 and 13 species) than Central province (14 and 4 species).

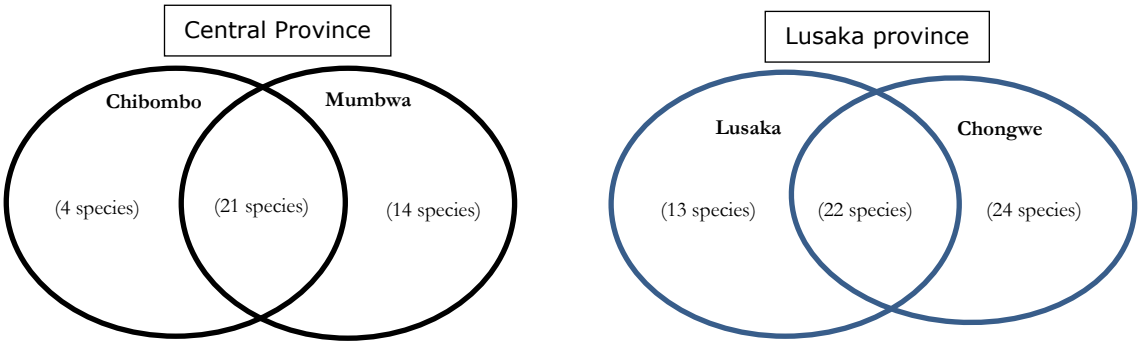

Figure 7. Venn diagrams showing overall intersection of bacterial species between Lusaka and Central province with a different number of uncommon species in each of the provinces.

We performed an anosim to determine whether the variation observed in the microbial communities among the samples can be attributed to sampling location and the processing and consumption variables.

Results in Table 3 show that clustering both sampling location and when the product is consumed explain a significant part of the variation in the observed bacterial community structure. It should be noted, however, that when the product is consumed also varied significantly by location, resulting in an autocorrelation in this analysis. In contrast, processing variables do not explain the variation in observed microbial community structure, nor do parameters related to the use of the product.

Table 3. Anosim analysis for impact of treatment variables such as location, processing and consumption on OTU tables. Variable, test statistic (R), degrees of freedom (Dfs) and exact p value (P) are given, unless the p value was smaller than 0.001, which is indicated by <0.001.

Variable	R	Dfs	P
Sampling location	0.208	4,87	<0.001
Province	0.293	2,87	<0.001
Cooking time	0.059	5,87	0.106
Hydrolysis time	0.072	4,87	0.051
Fermentation time	0.015	3,87	0.32
How consumed	0.035	4,87	0.107
Who consumed	0.035	4,87	0.241
Where consumed	-0.023	2,87	0.741
When consumed	0.129	4,87	<0.001

Aroma compounds in Munkoyo

To further characterize Munkoyo and its variations, aroma profiles of 15 samples covering all surveyed locations with different commonly used inoculum treatments were measured. Full aroma profiles are indicated in Figure 8. The most abundant compounds include hexane, ethyl acetate, 2-3 butanedione, acetic acid, 2, 3 pentanedione, ethanol, hexanal and 2-n-pentylfuran.

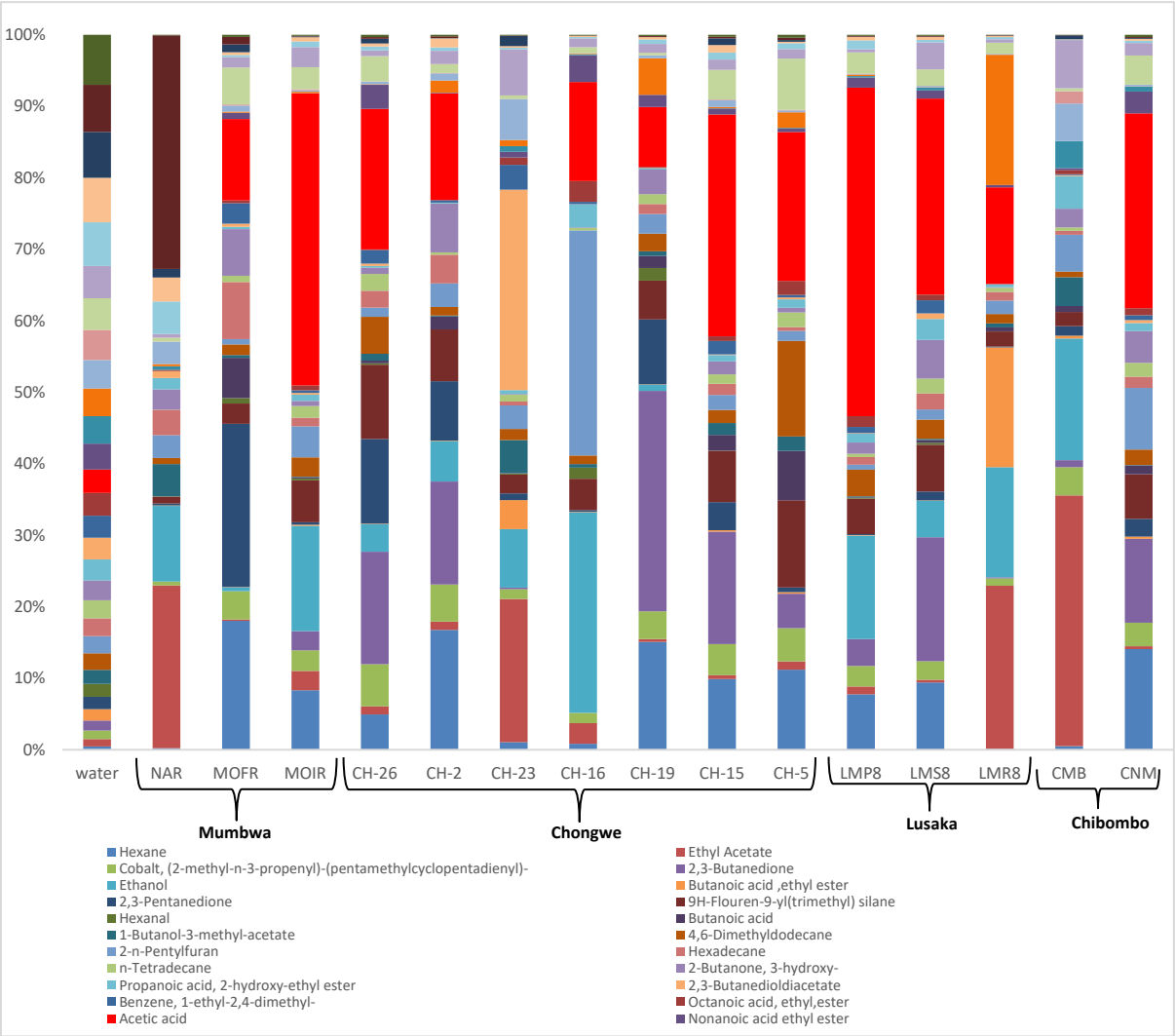

Figure 8. Aroma compounds in Munkoyo with acetic acid and 2,3-butanedione dominating in the samples.

The produced aroma compounds were aldehydes, esters, organic acids, alkanes, alkenes and alcohols as indicated in Table 4.

Table 4. Aroma compounds in Munkoyo from all surveyed locations with different commonly used inoculum treatments.

Aldehydes	Esters
2,3-Butanedione	1-Butanol,3--methyl-,acetate
2,3-Pentanedione	2,3-Butanedioldiacetate
3-hydroxy-2-Butanone	Acetic acid, 2-phenylethyl ester
2-n-Pentylfuran	Butanoic acid ,ethyl ester
Hexanal	Ethyl Acetate
Organic acids	Nonanoic acid ethyl ester
9-Hexadecenoic acid	Octanoic acid, ethyl,ester
Acetic acid	Propanoic acid, 2-hydroxy-ethyl ester
Butanoic acid	**Alkanes**
3-methyl- Butanoic acid,	4,6-Dimethyldodecane
n-Hexadecanoic acid	9H-Flouren-9-yl(trimethyl) silane
Octadecanoic acid	Hexadecane
Oleic Acid	Hexane
Tetradecanoic acid	n-Tetradecane
Alkenes	**Alcohol**
(5Z)-2,6,10-trimethyl-1,5,9-undecatriene	Ethanol
1-ethyl-2,4-dimethyl- Benzene	p-meth-1-en-8-ol
Cobalt,pentamethylcyclopentadiene	
Naphthalene	

Discussion

In this study we reported the results of a survey documenting the production practices and consumption patterns of Munkoyo from different regions in Zambia; assessed physicochemical properties and profiled the bacterial communities and the aroma compounds in Munkoyo, which linked variations in processing and consumption preferences to variations in bacterial communities involved in the spontaneous fermentation process; and determined the aroma profiles of a subset of samples.

The main variations in processing practices include time for cooking of the maize/water mix (cooking time), time for incubation of enzymes from *Rhynchosia* roots to allow degradation of gelatinized starch (hydrolysis time) and time allowed for fermentation by bacteria (fermentation time). The pH of Munkoyo ranged from 3.8 to 4.2 and TTA ranged from 0.2% to 0.8%. This low

pH and high acidity are known to reduce the proliferation of most pathogenic bacteria *(Kingamkono et al., 1999, Mbugua & Njenga, 1992)*. This may explain why there are seldom cases of pathogenic contamination in Munkoyo despite general poor sanitary conditions during processing procedures.

Surveys on consumption patterns showed that Munkoyo is mainly consumed as an energy drink by the entire population when hungry and at household level, whereas it is a social drink in urban areas. Processing practices and consumption patterns varied per sampling location. The fact that most rural communities consume Munkoyo as an energy drink when they are hungry and because it is easily prepared at household level with readily available raw material, make Munkoyo an ideal beverage to promote food security. This has also been observed for similar beverages like Kenkey, Mawe, Ogi and Akpan in West Africa *(Oyewole, 1997, FAO, 2011)*.

The profiling of bacterial communities revealed that the most dominant microbial species in this research include Streptococcaceae, Leuconostocaceae, Enterobacteriaceae, Lactabacillales, Bacillaceae, and Aeromonadaceae. The characterization of the microbial communities of the samples revealed high levels of diversity of bacteria within samples and high variation in bacterial community structure between samples. The Shannon index of up to 1.18 with an effective number of species of 3.44, indicates a relatively high diversity within bacterial communities. Like many other cereal-based fermented beverages, Munkoyo is largely spontaneously fermented, dominated by lactic acid bacteria *(Foma et al., 2012)*. Previous work on the microbial community structure of Munkoyo reported a similar bacterial composition and other studies evaluating the microbial communities of cereal-based fermentations reveal a great diversity of microbial communities with lactic acid bacteria being dominant *(Schoustra et al., 2013)*. This is in line with studies on cereal-based traditional fermented foods and gruels such as Ogi from Nigeria, Mawe and Akpan from Benin and Togwa from Tanzania *(Mugula et al., 2003, Sacca et al., 2012, Odunfa, 1988, Blandino et al., 2003, Hounhouigan et al., 1993)*.

Cluster analysis of factors that explain the variation in bacterial community structure in relation to processing variables and consumption patterns, revealed that how the product is consumed and sampling location determined consumption pattern and bacterial community structure, respectively. Processing variations of one hour cooking time, one day hydrolysis time and one day fermentation time exist, which apparently do not significantly alter microbial community composition. In previous work with only six Munkoyo samples *(Schoustra et al., 2013)*, sampling location was not identified as a driver for differences between microbial communities, and the suggestion was made that processing practices could be important. In the present study, based on 96 bacterial community profiles, we find no evidence for processing practices as major driver of bacterial community structure, but rather that sampling location is most important.

The action of bacteria on fermentable sugars produces aroma compounds. Acetic acid was the most dominant aroma compound observed in all samples analysed. This could be due to the presence of acetic acid bacteria in biofilms in the fermentation vessels forming a microbial community with other microbes that produce acetic acid *(De Roos & De Vuyst, 2018)*. Other researches show that the Acetobacteraceae family is characterized by their ability to metabolize carbohydrates, thereby releasing the corresponding products (aldehydes, ketones and organic acids) and oxidizing ethanol into acetic acid in aerobic conditions *(De Roos & De Vuyst, 2018, Mamlouk & Gullo, 2013)*. Munkoyo is mostly fermented in buckets or calabashes, which can produce biofilms that can harbor acetic acid bacteria. The fact that fermentation is spontaneous in open air facilitates the oxidation of ethanol into acetic acid. Acetobacteraceae being among the family of bacteria identified and ethanol among the aroma compounds produced in Munkoyo suggest the possibility of a quick conversion of ethanol into acetic acid by Acetobacteraceae. These findings highlight the fact that spontaneous fermentation constitutes diverse microbial communities, which can possibly lead to variation in sensorial attributes of the beverage like flavour and taste. To follow up on the relation between bacterial composition and aroma profiles, directed experiments using defined

mixes of bacteria could reveal what groups of bacteria are responsible for which type of aroma. These experiments are reported in Chapter 4.

The aroma compounds in the 15 samples from the different provinces under study include aldehydes, esters, alkenes, alkanes, organic acids and alcohols. This was not different from Gowe, a traditional malted fermented sorghum beverage from Benin, which contained groups of alcohols, aldehydes, organic acids, esters, hydrocarbons, furan and phenol as a result of spontaneous fermentation *(Vieira-Dalode et al., 2016).*

The micro-organisms found in Munkoyo and similar products are known to produce compounds with antimicrobial activities that act against some diarrhoeagenic bacteria *(Soro-Yao et al., 2014, Kort & Sybesma, 2012).* These antimicrobial effects have been confirmed by studies that lactic acid bacteria are effective in reducing constipation severity and improve bowel movement frequency in constipated but healthy people after consumption of fermented foods containing a specific *Lactobacillus casei* strain *(Ouwehand et al., 2002).* Further research could formalize these general findings for Munkoyo to establish potential health benefits of Munkoyo. The research could also include the addition of microbial specific strains known to have probiotic properties, such as *Lactobacillus rhamnosus (Kort & Sybesma, 2012),* and the inclusion of bacteria that produce vitamins such as vitamin B12 and vitamin K *(Wolkers – Rooijackers et al., 2018).*

Other further research to determine the link between specific microbial communities and the aroma profiles responsible for the sensory attributes in Munkoyo should be undertaken. Based on the fact that we detected wide variation in the microbial communities underlying Munkoyo fermentation, we expect that different combinations of different lactic acid bacteria would be able to generate the desired texture and aroma profiles. This future research is essential to design appropriate mixes of micro-organisms for the production of starter cultures for commercial production of fermented foods that optimally meet the desired sensorial quality characteristics.

CHAPTER – THREE

The effect of different processing methods on the microbial composition in cereal-based fermented Munkoyo beverage

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Abstract

Cereal-based traditionally fermented beverages are important in attaining food security in Africa**.** Differences in their processing impact sensory and nutritional quality. The effect of variations in processing methods on the microbial community composition of a cereal-based fermented beverage in Zambia, called Munkoyo, was analysed in products from three agro-ecological zones. In Choma, Munkoyo was processed from maize grits, and *Rhynchosia* root extract was added to the cooked porridge as a source of enzymes. In Nyimba, maize meal was used and *Rhynchosia* roots were immersed in the porridge overnight. In Kitwe, maize meal was cooked until it caramelised, followed by immersion of *Rhynchosia* roots. Titratable acidity of Munkoyo was between 0.3% to 0.5% weight/volume, irrespective of processing method, and the final pH ranged between 2.5 and 3.5, with the lowest values recorded in Nyimba. Processing method did not completely cluster the DNA of the microorganisms together as a monophyletic group, and significantly affected the operational taxonomic unit (OTU) composition (anosim, $R_{3,13} = 0.407$, $p<0.05$). The average Shannon indices, which indicate ecological diversity, were: Choma 1.14 ± 0.64, Nyimba 1.58 ± 0.23, and Kitwe 1.07 ± 0.95. This work suggests that various combinations of bacterial species can produce Munkoyo that addresses local preferences.

Introduction

Many types of cereal-based fermented beverages are produced in Africa with varying product characteristics. Examples include Togwa from Tanzania, Maheu from Zimbabwe and South Africa, Mawè from Benin and Munkoyo from Zambia and the Democratic Republic of Congo (DRC) *(Zulu et al., 1997, Hounhouigan et al., 1993, Mugula et al., 2003).* Most of these beverages are prepared by applying similar basic processing steps like cleaning and milling of the raw materials. Generally the preparation of these products is still a traditional family art with an uncontrolled fermentation process by diverse microbial communities. The composition of these microbial communities largely determines key product properties *(Smid & Hugenholtz, 2010, Macori & Cotter, 2018).* Variations in microbial communities may result in variations in product quality, taste, acceptability and microbial stability of the products *(Sanni, 1993).* The common processing method for cereal-based fermented beverages involves cooking of raw materials to gelatinize the starch, addition of a source of enzymes to hydrolyse the gelatinized starch into fermentable sugars and finally spontaneous fermentation *(Kitabatake et al., 2003, Blandino et al., 2003, 2014).* Most cereal-based non-alcoholic and alcoholic beverages in Africa are prepared by using either sorghum or finger millet malt as a source of enzymes for the degradation of starch into fermentable sugars *(Gadaga et al., 1999).*

Individual case descriptions of the processing methods of many cereal-based beverages are available but their processing protocols have not been systematically analysed and reviewed. Optimizing the sensory attributes while ensuring the microbiological safety and stability of the product can be complex *(Masood et al., 2018).* Differences in the cereals used and the specific processing steps of these beverages exist from one region of Africa to the other, and for some of the final products the microbial communities that underlie fermentation have been characterized. For instance, Maheu in Zimbabwe and South Africa are made from dry maize meal as basic ingredient, whilst Ogi in Benin and Nigeria is produced by first steeping whole maize grains in earth ware or a plastic bucket for one to three days *(Soro-Yao et al., 2014).* For Ogi, the grains contain

various microbial species, such as fungal species belonging to the genera *Aspergillus* and *Penicillium*. Upon steeping, these fungi get replaced by bacteria and yeast with the resulting microbial population largely consisting of species of lactic acid bacteria belonging to genera *Lactobacillus, Streptococcus* and *Leuconostoc* and the yeast *Candida krusei* (*Odunfa & Adeyele, 1985).* Specific variations have been observed in some processing methods such as for Kwete from Uganda, in which sourdough is roasted on an open fire to give a golden brown colour and the typical Kwete flavour. Variations in processing methods of Kwete contribute significantly to variations in the microorganisms involved in the fermentation *(Muyanja & Namugumya, 2009).*

Here, we focus on the traditional processing of Munkoyo, a commonly produced and consumed indigenous fermented beverage found in Zambia and the Democratic Republic of Congo (DRC). For Munkoyo, processors follow similar general steps, with variations on the specific features of each processing step such as cooking maize porridge, the addition of *Rhynchosia* roots or root extract as a source of enzymes *(Zulu et al., 1997, Simwamba & Elahi, 1986, Foma et al., 2013)* and fermentation for 24 up to 72 h in a plastic container. Cooking the maize meal into porridge is a crucial processing step that primarily gelatinizes the starch and makes the beverage palatable. Starch gelatinization disrupts the inter and intra molecular hydrogen bonds between starch chains and causes swelling as the water is gradually absorbed, thereby facilitating the activity of α and β amylases in *Rhynchosia* roots, which hydrolyse starch into fermentable sugars.

The fermentation process leads to the characteristic flavour and taste of Munkoyo due to the action of lactic acid bacteria, mainly *Lactobacillus*, *Streptococcus* and *Leuconostoc* species. As fermentation proceeds, the pH of the Munkoyo beverage decreases to around 3.3 - 3.7*(Schoustra et al., 2013).*

The aim of this research is to assess the effect of variations in processing methods on the bacterial community composition that underlies the fermentation of Munkoyo beverage. Since the bacterial community structure largely determines the sensory and nutritional quality of Munkoyo, this is an essential step towards enabling the optimisation of Munkoyo processing. Specifically, we assess the

effect of variations in processing methods between three regions in Zambia that have their own

Figure 1. The regions of Zambia where the experiments on variations in Munkoyo processing were performed, namely Low rainfall veld region I Choma, Middle rainfall veld region II Nyimba and High rainfall veld region III Kitwe.

distinct preparation method. In each of these regions, the production process of Munkoyo is an artisanal skill. Differences in production procedures have led to regional variations in the attributes of the beverage. Documenting the processing steps and ingredients in the production of Munkoyo can conserve the art of making this traditional fermented beverage as well as serve as a knowledge base to standardize processing, improve sensory and nutritional properties to increase product satisfaction and health benefits to consumers. In the end, this may facilitate commercialisation of this popular beverage in urban areas, to build value chains to improve the livelihoods of small-scale rural producers.

Materials and methods

Study areas. Experiments were performed in selected areas in Zambia (Figure 1), which were chosen because at each location a specific processing method is used. The areas are in different agro-ecological zones of Zambia with particular climatic conditions, namely low rainfall veld (Choma I), middle rainfall veld (Nyimba II) and high rainfall veld (Kitwe III) *(JAICAF, 2008).*

Field experiments. The field experiments investigated the effect of variation in processing methods of Munkoyo on bacterial community composition in the final product. We recorded the exact characteristics of three distinct processing methods at the three locations. The variant of Munkoyo in Choma is also referred to as Chibwantu beverage, and is prevalent in the southern part of Zambia. The variant of Munkoyo in Nyimba is considered the most common way of processing Munkoyo as known by the general Zambian public in large urban areas. The variant of Munkoyo in Kitwe is specific to the Copperbelt province. Parameters of variation among these three methods of processing included the type of maize flour used, the type of roots used for the hydrolysis of the maize porridge, the vessel in which the fermentation was carried, the time allowed for cooking and the time allowed for fermentation. In all three processing methods, 10 L of water was heated to at least 60°C. Next, 1500 g maize flour or grits was added slowly whilst stirring to avoid settling or lump formation. The porridge was left to boil for a maximum of 120 min until complete gelatinization. In Nyimba the gelatinized porridge was let to cool to 45°C - 50°C before adding 150 g of dried and shredded *Rhynchosia* root. In Choma, 150 g of dried and shredded *Rhynchosia* root were soaked in 1L water for 20 mins and collected the extract which was added into gelatinized porridge at temperatures between 45°C - 50°C. In Kitwe, 150 g of fresh *Rhynchosia* roots were shredded and soaked in 1L water for 20 mins and poured the mixture of the root and extract in gelatinized porridge at temperatures between 45°C - 50°C. The mixture of gelatinized porridge and the roots or extract was left to hydrolyze for a minimum of 4 h to a maximum of 24 h. In the case where roots were added, they were removed after hydrolysis, and spontaneous fermentation proceeded for up to 72 h.

Physicochemical analysis. The temperature during cooking and inoculation of *Rhynchosia* roots was measured with a laboratory thermometer. Titratable acidity was determined by titrating 10 cm^3 of the sample against sodium hydroxide with phenolphthalein as an indicator *(Foma et al., 2012).* The pH value was recorded every 12 h using a portable pH meter (HI 151 HANNA instruments) for 72 h from the time the roots were added to the porridge.

Bacterial community characterization. The composition of the bacterial communities was characterized by 16S amplicon sequencing using bacterial DNA that was extracted from all samples. DNA extraction was as follows. One ml of Munkoyo sample was spun down at 1,880-g-force after which the supernatant was discarded. Five hundred µl TESL, 10 µl mutanolysin solution and 100 µl lysozyme solution were added to the pellet and incubated at 37°C for 60 min with slight shaking. Five hundred µl GES reagent was added, followed by cooling on ice for 5 min. Two hundred fifty µl of cold ammonium acetate solution was mixed gently and held on ice for 10 min before spinning and collecting the supernatant. The supernatant was purified with chloroform-2-pentanol by mixing 1:1, spinning down at 12,000 rpm and collecting the supernatant. DNA was precipitated by adding 0.1 volume of 3 M sodium acetate followed by 2.5 volumes of 100% ethanol and overnight storage at -20°C. Thereafter the mixture was spun for 20 min at 12,000 rpm at 4°C after which the supernatant was carefully decanted without losing the DNA-pellet. Next the salts were washed out by adding 1 ml of cold 70% ethanol without vortexing, but spinning for 10 min at 12,000 rpm at 4°C. Finally, the supernatant was decanted, the DNA-pellet air dried for 10 min at room temperature and dissolved in 10 mM Tris pH 7.5(Schoustra et al., 2013)**.** The DNA of the bacterial communities was analyzed using 16S amplicon sequencing *(Neto et al., 2018., Groenenboom et al., 2019)*. For further data processing and statistics the QIIME pipeline *(Caporaso et al., 2010),* paired-end reads were joined using join_paired_ends.py (with minimum overlap 10 base pairs) after which sequences were trimmed and filtered using cutadapt (v1.11 -q 20, -m 400, Martin 2011) using the known primer sequences CCTACGGGNGGCWGCAG and GACTACHVGGGTATCTAAKCC to trim both sides of the sequence. These trimmed sequences were then checked for chimeras, using uchime (v4.2.20, gold database). Sequences with a lower chimera score than 0.28 were retained. After these trimming and filtering steps sequences were clustered into operational taxonomic units (OTUs) after quality check using pick_open_reference_otus.py (-s 0.1, -enable_rev_strand_match TRUE, -align_seqs_min_length 75, -pick_OTU_similarity 0.95). Taxonomy of the resulting OTUs was assigned to representative

sequences using the Greengenes (v13.5) rRNA database. This algorithm gives a representative sequence for an OTU, which was used to perform a local blast using the gold database from uchime. The taxonomy from the top BLAST hit was used for further data processing. To further test whether location significantly affects the community, i.e. OTU table, an ANOSIM was performed, using compare_categories.py (QIIME method anosim -n 999) *(Caporaso et al., 2010)*. For this analysis we used the first of multiple rarefactions with a subsampling of 14,000 reads using the multiple_rarefactions.py and beta_diversity.py scripts. Next the multiple rarefactions were used to produce a consensus tree (with upgma_cluster.py and consensus_tree.py).

Data analysis. The Statistical Package for Social Sciences (IBM SPSS statistic 23) and Microsoft Excel 2016 were used to analyse the results. Grouping of microbial communities based on processing method was performed using specific commands from QIIME *(Bik et al., 2016)*.

Results

*Processing methods*In each of the three regions, we documented the details of the processing methods used. Results are visualized in Figure 2.

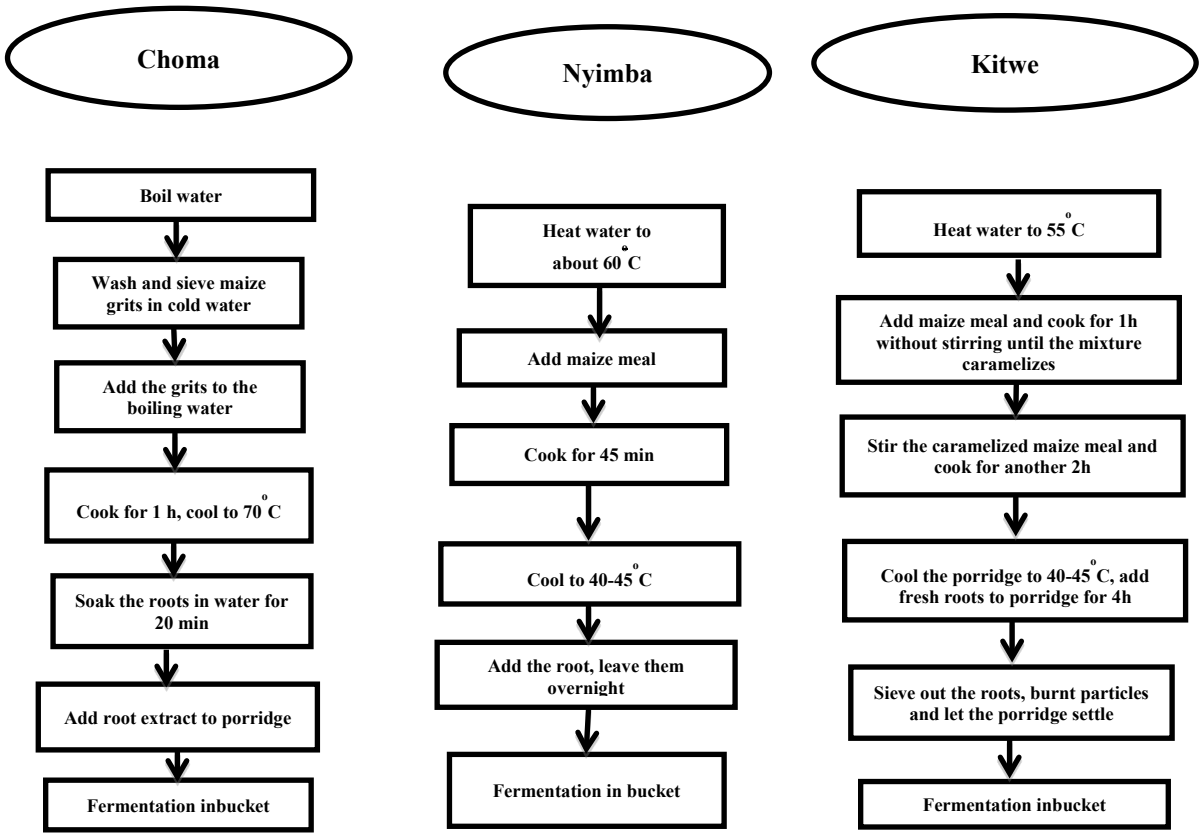

Figure 2. Processing methods of making Munkoyo in selected regions of Zambia, namely Choma, Nyimba and Kitwe.

The common steps in Munkoyo processing included mixing of maize meal or maize grits with 10 L of water and allowing the mixture to cook for at least 45 min. After this, *Rhynchosia* roots or millet malt were added for least 12 h for hydrolysis of starch followed by a fermentation period of at least 24 h. The cooking was essential to gelatinize the starch prior to hydrolysis to produce fermentable sugars. In general, the gelatinized porridge comes into contact with *Rhynchosia* roots at about 45°C. A 24 h fermentation period was sufficient for the beverage to develop its characteristic sensory attributes, although in practice fermentation time can go on up to three days. Key features of the different processing methods are as follows. Munkoyo from Choma was made using maize grits as raw material and root extract for hydrolysis. In Nyimba, Munkoyo was made by cooking for one hour and incubating the immersed *Rhynchosia* roots overnight. In Kitwe, Munkoyo was made from

maize flour that was allowed to caramelize by extended cooking without stirring to add a slightly burnt flavour and colour to Munkoyo.

Physicochemical properties

For each of the three methods, four or five samples of the final product were analyzed. Titratable acidity of Munkoyo samples was statistically different, ranging between 0.3% to 0.5% weight/volume irrespective of the processing method used (Figure 3a), with analysis of variance (ANOVA; $F_{1,12}$= 8,978, p= 0.035). The final pH of Munkoyo ranged between 2.5 and 3.5 for measurements taken every 12 h during processing (Figure 3b). This pH trajectory was different with respect to the processing method used, with the Nyimba processing method yielding rapid acidification leading to the lowest pH value. These differences were statistically significant with the analysis of covariance (ANCOVA; $F_{1,14}$= 184.48, p<0.001).

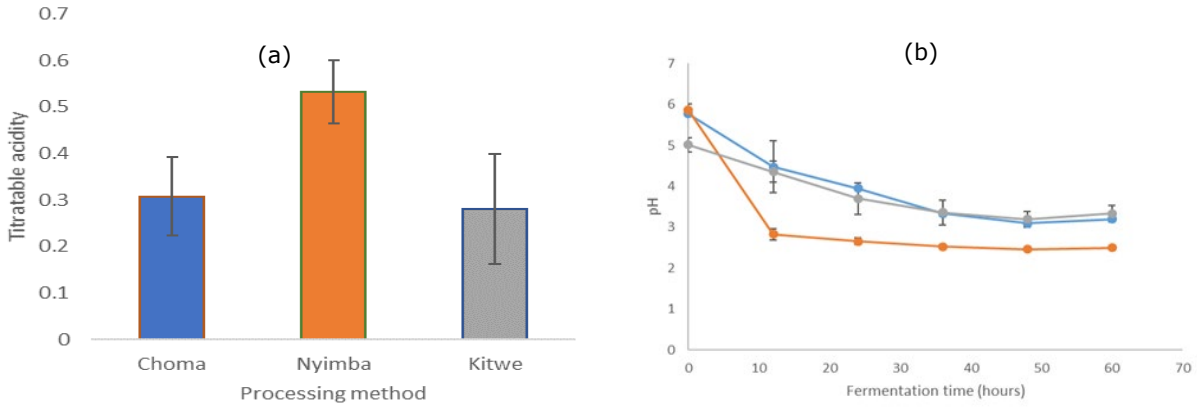

Figure 3. (a) Titratable acidity of Munkoyo beverage based on samples taken at the end of processing from different processing methods. The values shown are an average of all samples taken per processing method. (b) pH measured every 12 h during the fermentation of each processing method with the start of fermentation defined as the moment the roots were added. Error bars show standard deviation.

Bacterial community composition and structure

The analysis of the microbial community composition based on 16S rRNA amplicon DNA sequencing, combining all samples, is presented in Figure 4. The most abundant families of lactic acid bacteria were Streptococcaceae (34%), Lactobacillaceae (30%), Enterococcaceae (5%) and Leuconostocaceae (4%), amounting to 73% of all identified bacterial operational taxonomic units (OTUs).

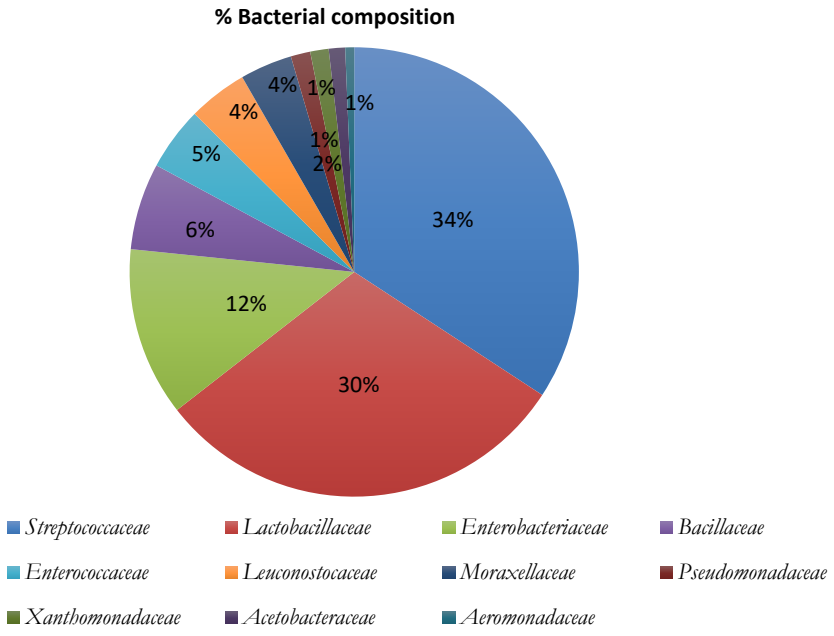

Figure 4. Overall microbial composition of all combined Munkoyo samples differentiated at the taxonomic level of bacterial families based on 16S amplicon sequencing using full community bacterial DNA.

Figure 5 shows bacterial community profiles of all samples and a dendrogram to represent the clustering. Munkoyo from Choma was largely dominated by Streptococcaceae and Lactobacillaceae, Munkoyo from Nyimba by Enterobacteriaceae, Streptococcaceae, Moraxellaceae and Leuconostocaceae and Munkoyo from Kitwe by Lactobacillaceae. The dendrogram further revealed that Munkoyo processed using the same processing method does not completely cluster together as a monophyletic group and that processing method significantly affected the OTU composition, with Nyimba being different from the other two processing localities (anosim, $R_{3,13} = 0.407$, $p<0.05$). Based on the bacterial community composition, the Shannon index for ecological

diversity for each sample was calculated. Shannon diversity index mathematically expressed as $H = -\sum P_i \ln P_i$, measures diversity of species in a community. The higher the Shannon index the more diverse the species are in a community. The average Shannon indices and standard deviations per processing method were: Choma 1.14 ± 0.64, Nyimba 1.58 ± 0.23, and Kitwe 1.07 ± 0.95.

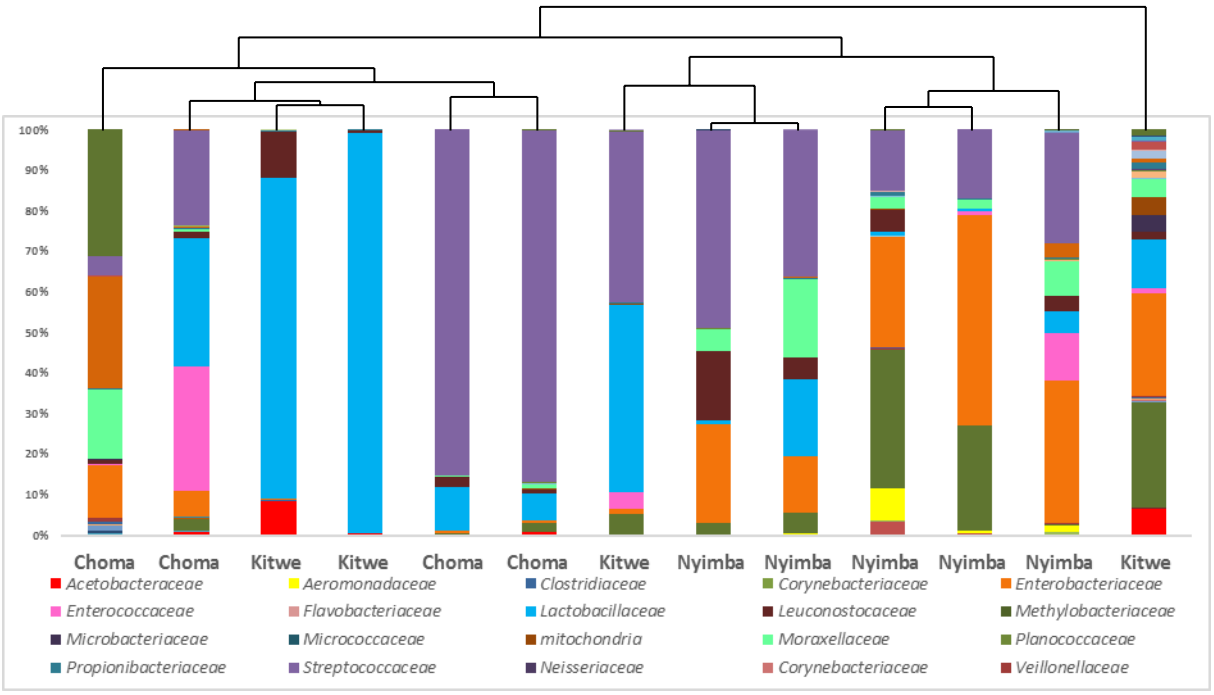

Figure 5. Bacterial community structure for each sample taken. Different colours represent different bacterial families. Each bacterial family contains multiple unique types of (OTUs). The dendrogram on the top of the figure is based on the level of similarity between the bacterial communities in each sample. Clustering of the bacterial communities based on processing method explains significant parts of the variation in the community structure.

Discussion and conclusions

The main goal of this research was to determine the impact of variations in processing methods of Munkoyo on titratable acidity, pH and bacterial community structure. We found that Nyimba processing method had different physicochemical properties than other processing methods that yielded low pH and high titratable acidity. The distribution of the Shannon indices was relatively the same across categories of processing methods, indicating that processing methods do not lead to significant variations in microbial diversity. First we documented the specifics of the processing methods in the three study areas in Zambia. Methods for making Munkoyo differ mainly with respect to the state of raw material, time allowed for cooking and the way in which the *Rhynchosia*

roots are applied. In Choma, the product is called Chibwantu. It was processed from maize grits, and *Rhynchosia* root was immersed in water to extract the enzymes rather than immersing the whole root in the porridge. In Nyimba, maize meal is used as starting material and the *Rhynchosia* roots are immersed in the porridge overnight. In Kitwe, maize meal is used as starting material with extended cooking to caramelize the starch to add colour and flavour, and the *Rhynchosia* roots are equally immersed in the porridge.

The ranges of titratable acidity found are comparable to those reported by other researchers *(Sahlin & Nair, 2012)*. A study of a cereal-based beverage in Zimbabwe called Masvusvu showed an increase in total titratable acidity from 0.13 to 0.67% weight/volume and a pH drop from 6.1 to 4.0 after 8 h of fermentation *(Zvauya et al., 1997)*. The final pH observed in Munkoyo ranged from 2.5 to 3.5, with the Nyimba processing method resulting in the lowest pH. This pH range is consistent with other studies on Munkoyo *(Zulu et al., 1997)* and lower than the common pH ranges observed in other cereal-based fermentations as for instance reported for Oshikundu, a cereal-based beverage in Namibia with a pH range of 3.3 to 3.7 *(Misihairabgwi & Cheikhyoussef, 2017)*.

The characterization of the bacterial community compositions showed that overall Streptococcaceae, Lactobacillaceae, Enterococcaceae and Leuconostocaceae were the most abundant types of lactic acid bacteria in Munkoyo. This was expected as research has shown that lactic acid bacteria dominate most cereal-based fermentations. The common genera of fermenting bacteria (i.e. *Leuconostoc, Lactobacillus, Streptococcus*, *Pediococcus, Micrococcus* and *Bacillus*) are frequently reported for cereal-based fermented products *(Blandino et al., 2003)*. *Lactobacillus* species are known to produce a variety of metabolites, including lactic acid and acetic acid, which lower the pH to levels that are inhibitory to many competing microorganisms such as psychrotrophic pathogens *(Sanni, 1993)*. Siroli et al. (2015) studied *Lactobacillus plantarum* strains CIT3 and V7B3 on apples and lettuce. Fermentation with these two *Lb. plantarum* strains showed a reduction in pH, which increased both the safety and shelf-life *(Siroli et al., 2015)*. The rise in titratable acidity and drop in pH improves food safety and extends the shelf life of many fermented cereal-based products. So,

within the pH range of Munkoyo, proliferation of most pathogenic bacteria is reduced or even completely inhibited, thus increasing the safety of the beverage. This might explain why reported cases of contamination with food borne pathogens in Munkoyo are seldom, even though the beverage may be processed in poor hygienic conditions as potentially occurs in resource-poor households.

- The fact that the final pH in Munkoyo often was lower than in other fermented cereal-based products, suggests the presence of greatly acidifying bacteria but is also probably be due to the low buffering capacity of cereals because of their low protein content *(Oluwole & Bolarinwa, 1986)*. This could be related to the microbial community composition coming from the *Rhynchosia* roots from high rainfall areas region III of Zambia, which have a low soil pH due to soil leaching *(Chabala et al., 2014)*. Moreover, the microbial soil community is known to be affected by management practices and soil organic matter content, which are main differentiating features between agro-ecological zones *(García-Orenes et al., 2013)*. Measurements of soil pH and organic matter when harvesting *Rhynchosia* roots could be part of further research to ascertain the link between the soil characteristics and the microbial community composition of the *Rhynchosia* root collected from such soils. Pilot work analyzing the microbial community structure from a *Rhynchosia* root extract showed that lactic acid bacteria were not abundant *(Van Damme, 2017)*. After constructing a clone library, 74 sequences were identified using BLAST. *Pseudomonas*, *Enterobacter* and *Enterococcus* species formed the majority of bacteria residing on the dried roots. Minor species on the surface of the roots were *Klebsiella*, *Pantoea*, *Stenotrophomonas*, *Acinetobacter* and *Brevibacterium*.

As the level of titratable acidity and final pH of the different processing methods yielded different outcomes, an effect of processing method on product properties was expected. This assumption was supported by the significant variations in bacterial community composition related to processing practices. This suggests that various combinations of bacteria can yield desired product properties. Several factors may have contributed to the observed variations. Firstly, the *Rhynchosia*

roots used for fermentation could contain highly different bacterial communities, which are specific to the processing location/region; in all cases roots were used that were collected locally. In future research, the community composition of bacteria associated with the roots should be determined before and after processing to assess how different processing methods affect microbial community composition. Further, environmental factors such as temperature and soil properties *(Xue et al., 2018)*, should be taken into account as potential drivers of variation.

Our results have implications for the development of starter cultures for standardized processing methods for Munkoyo. Such starter cultures could be required when the trend of diminishing *Rhynchosia* shrubs continues. The dissimilar composition of bacterial communities in different regions indicates a reason for the variations in the commonly perceived sensorial properties of Munkoyo from different regions. This work suggests that various combinations of bacterial species could be used to produce Munkoyo to address local preferences. Further research on functional properties of individual bacterial species and their combinations is recommended, taking the nutritional quality as a major objective.

CHAPTER – FOUR

Production of aroma compounds by complex bacterial communities in Munkoyo*:* a cereal-based fermented beverage

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Manuscript in preparation

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Abstract

Aroma compounds found in the fermented cereal-based beverage Munkoyo, originate mostly from the community of bacteria driving the fermentation process. In this study, single strains and combinations of strains of the species *Acetobacter orientalis (Ac), Lactobacillus helveticus* (*Lb*), *Lactococcus lactis* (*Lc*), *Leuconostoc mesenteroides* (*Le*), *Streptococcus thermophilus* (*St*) and *Weissella confuse* (*We*) were used to produce Munkoyo to determine the most influential bacteria or groups of bacteria in the production of aroma compounds in Munkoyo. The pH of the product was monitored for 48 h of fermentation and aroma compounds produced were analysed. The pH of the fermentable raw material did not significantly change when inoculated with most of the single strains and combinations of strains. However, with single strain inoculations of *St*, *We* and with the following combinations of strains *(Lb-Lc-St-We*, *Ac-Lb-Le-St-We, Ac-Lb-Lc-Le-St-We*), the pH dropped below 6, indicating acidification due to production of organic acids causing a sour taste of the product. Various groups of volatile aroma compounds (esters, alkanes, alkenes, aldehydes, organic acids and alcohols) were identified in Munkoyo. Furthermore, it was demonstrated that spontaneously fermented Munkoyo and Munkoyo made from the combination of all six species of bacteria had a similar composition of aroma compounds. This implies that the complete mixture of microbes (*Ac-Lb-Lc-Le-St-We*) can potentially be used as starter culture for Munkoyo production.

Introduction

Cereals are a major part of the human diet and are rich in carbohydrates with low abundance of certain essential amino acids like lysine. Fermentation is an efficient and economical way of improving the nutritional value, sensory properties and functional qualities of most cereal-based products *(Blandino et al., 2003).* Spontaneous fermentation of cereals into non-alcoholic cereal-based beverages like Munkoyo found in Zambia and the Democratic Republic of Congo (DRC) is one way of utilizing cereals with improved sensory attributes. During the fermentation process, lactic acid bacteria transform fermentable sugars into organic acids and aroma compounds, yielding the desired sour taste and flavour of the beverage *(Mukisa et al., 2017).* The beverage is popular for its refreshing taste and aroma. It is consumed as an energy drink during long days of field work as well as during social gatherings and special ceremonies. The production process is based on local knowledge and takes place at household level. Finally, the product plays an important role in contributing to the livelihoods of many people through enhanced food security and income generation *(Misihairabgwi & Cheikhyoussef, 2017).*

Although variations in the processing of Munkoyo exist, there are fundamental similarities in how it is produced. It is generally processed from water, maize flour and *Rhynchosia* roots as a source of enzymes to degrade starch and possibly the bacteria fermenting the beverage. At the beginning of the process, maize flour and water are cooked into porridge for utmost 1 hour depending upon the quantity of Munkoyo being prepared. This cooking process allows starch gelatinization, which increases the viscosity of the mixture but also sterilizes the porridge. After cooling the porridge to about 45°C, *Rhynchosia* roots are added and the mixture is incubated for 48 hr at ambient temperature to allow hydrolysis by amylases that break down the starch into fermentable sugars like glucose, maltose and maltotriose, and ultimately reduce the viscosity of the gelatinized starch *(Foma et al., 2012).* Previous work has shown that a microbial community consisting of at least six different species of lactic acid bacteria (LAB) is responsible for the fermentation *(Schoustra et al., 2013).*

During the fermentation process, the Munkoyo aroma is formed and it is considered to be intense enough when the pH is decreased to around 3.5 - 4.5 *(Zulu et al., 1997)*. The production of organic acids and volatile compounds in cereal-based fermented products is driven by the activity of a community of LAB, which metabolizes fermentable sugars and other compounds like citrate to produce flavour-related compounds. A very good example is the natural occurrence of *Enterococcus faecium* in cheese capable of metabolizing citrate to pyruvate producing compounds with aromatic properties like diacetyl, acetoin and 2,3 butanediol *(Martino et al., 2016)*.

The activity of bacteria depends on the composition of the food substrate and environmental factors such as temperature, pH and osmolarity. In addition to primary metabolites, microbial communities produce a variety of volatile aroma compounds. Aroma compounds usually consist of many volatile and non-volatile components. Non-volatile compounds contribute mainly to the taste of the product, whilst volatile compounds influence both taste and aroma *(Longo & Sanromán, 2005)*.

The fact that Munkoyo production relies on spontaneous fermentation implies that the composition of the microbial communities differs between fermentation batches. Therefore, the production process potentially leads to diverse aroma compounds. The outcome of such processes is unpredictable because the inoculum is determined by the composition of microbial load of the fermentable raw material. In standardized products, the formation of reproducible aroma profiles depends on the activity of specific microbial communities to produce desired aroma compounds. This has been observed in the use of LAB starter culture during cereal dough fermentation, essential to produce a product with known sensory characteristics made from standardised steps and guaranteed product uniformity *(Soro-Yao et al., 2014)*.

Although the main groups of bacteria responsible for Munkoyo fermentation may be known, it is unclear which species of bacteria or combinations of bacterial species are the main drivers in the production of the Munkoyo aroma. Development of a starter culture with desirable properties can

be identified and applied to ensure reduction in processing time, consistent product quality and safety. However, selecting an appropriate starter culture requires understanding of the microbial diversity of the product and the roles played by specific organisms.

The aim of this study is therefore to identify the bacterial species responsible for the production of aroma compounds in Munkoyo and to further evaluate the individual effects of the most common bacteria found in Munkoyo in the production of aroma compounds.

Materials and methods

Culturing the bacteria

Strains of *Acetobacter orientalis* DSM 15550, *Lactobacillus helveticus* DSM 20075, *Lactococcus lactis* DMS 20481, *Leuconostoc mesenteroides* DSM 20484 *Streptococcus thermophilus* DSM 20617 and *Weissella confuse* DSM 20196 were obtained from Leibniz-Institute – DSMZ (Germany). The bacteria were cultured and grown on MRS agar plates (CM0361 Thermo scientific) under aerobic conditions, except for *Lactobacillus helveticus,* which was grown under anaerobic conditions. This was done by transferring single colonies of bacteria from the agar plates to M17 broth medium (CM0817 Thermo scientific) and incubated for 2 days until a turbid suspension was observed. An aliquot of 5 ml of the bacterial suspension was transferred into cryo-tubes to make a final concentration of 20% (v/v) glycerol before snap freezing with liquid nitrogen and stored at -20^{o}C until further use. For further use; frozen cultures were defrosted and 100-times diluted in fresh broth. Subsequently, the broth cultures were incubated again for 2 days at 30°C. The full-grown cultures were centrifuged (*Eppendorf 5430*R) at 1878- g-force for 15 min. The supernatant was then discarded and sterile Phosphate Buffered Saline (PBS buffer) was added to re-suspend the cells. PBS contains sodium chloride (80 g/L), sodium hydrogen phosphate (14.1 g/L), potassium chloride (2.0 g) and potassium hydrogen phosphate (2.4g/L) and the pH is adjusted to 7.4. The five tubes with the cell

suspensions of each species were subsequently put on ice before mixing in an equal volume ratio of 1:1:1:1:1.

Preparation of Munkoyo

For each batch, 152g of maize meal was mixed with 1L water at 55°C. With continuous stirring, the porridge was boiled for 30 minutes until gelatinisation was observed by visual inspection. The porridge was then cooled to 45°C before adding approximately 200g of small shreds of *Rhynchosia* roots, which were previously sterilized by 70% hydrogen peroxide to inactivate all microbes present on the surface of the root based on the plate count done on sterilized treatment that confirmed the effectiveness of the sterilization process. The enzymes from the *Rhynchosia* roots were allowed to hydrolyse the gelatinized starch for 4 hr before inoculating with bacteria.

Inoculation and fermentation

A mixture containing all six strains, mixtures with a single omission of one strain at a time (at a ratio of 1:1:1:1:1) and all strains as single cultures were used as outlined in the experimental set-up (see figure 1). As a positive control, Munkoyo was prepared by the traditional method using non-sterilized *Rhynchosia* roots. As a negative control only sterile broth was added to the porridge. In all cases a total of 1 ml of broth was used to inoculate 50 ml tubes of hydrolysed porridge. Fermentation was allowed in the incubator at 30°C during which pH was recorded every after 24 h to monitor the progress of the fermentation process. Fermentation was stopped after 48 h by snap freezing the samples in liquid nitrogen.

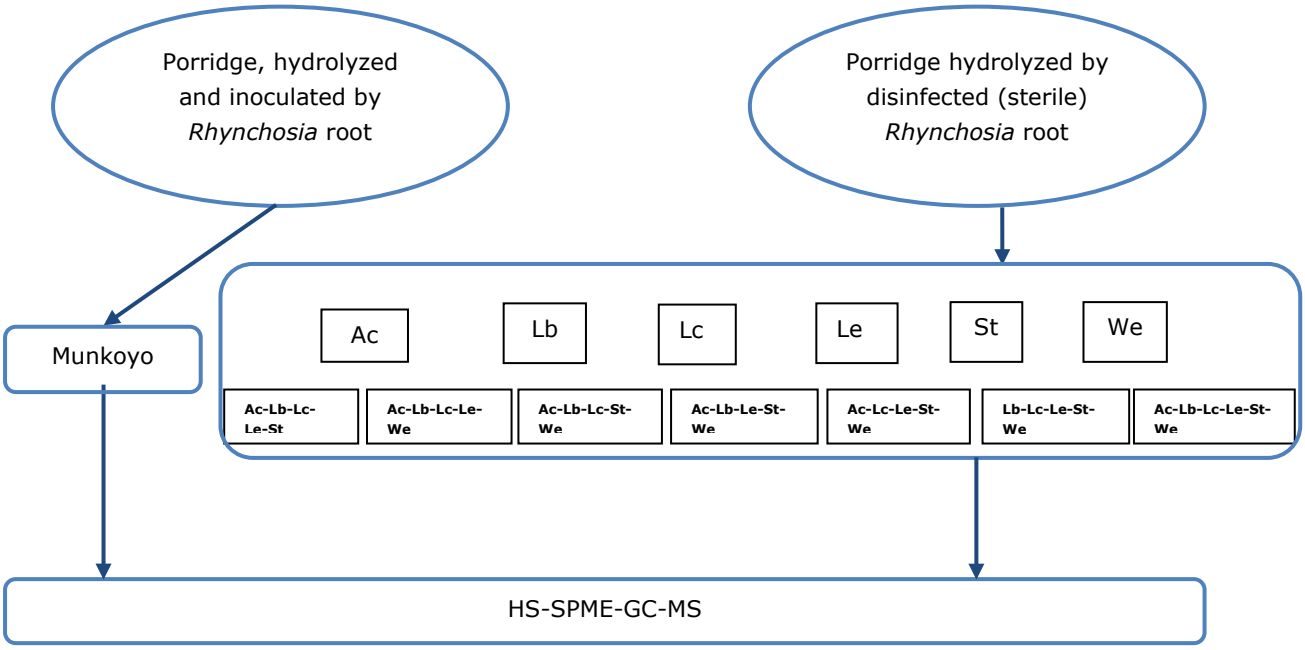

Figure. 1 Schematic overview of the experimental set-up. *Acetobacterorientalis(Ac)*, *Lactobacillus helveticus (Lb)*, *Lactococcus lactis (Lc)*, *Leuconostoc mesenteroides (Le)*, *Streptococcus thermophilus (St)* and *Weissella confusa (We).* The porridges were made and inoculated with disinfected *Rhynchosia* roots except for Munkoyo.

HS-SPME-GC-MS

After fermentation, the samples were analysed for volatile compounds by Gas Chromatography – Mass Spectrometry (GC-MS). The samples were first transferred to sealed glass vials and subsequently heated for 5 min at 60°C, during which they were agitated twice for 5 sec. After this, extraction of volatile compounds in the headspace followed for 10 minutes using a grey Solid Phase Micro Extraction (SPME) fibre (Car/DVB/PDMS *Thermo electron corporation*, Austin TX 78728 USA). Next, the fibre was brought into the injection port at which the sample was released for 5min using split-less mode at an inlet temperature of 250°C. The sample was carried through the Stabilwax-DA-Crossbond-Carbowax-polyethylene-glycol column (25 m length, 0.25 mm ID, 0.5 µm df) by helium as mobile phase flowing at 1.0 $ml.min^{-1}$. The initial temperature of the oven was 40°C, at which it was kept for 2 min. Next, the temperature was increased by 10°C per minute to 240°C at which it was kept for a final 5 min. To determine the unknown compounds, the mass spectrometer was set on full scan mode (m/z 33-250 at 3.0030 scans per second ~ 700 anu/s). The

probability limit for the identification of compounds was set on 70%; compounds that were found with lower probabilities were ignored.

Data analysis

Peaks from GC-MS were annotated using Chromeleon 7.2 software to obtain retention times and peak areas of the aroma compounds found in Munkoyo. The ICIS algorithm was used for peak integration and NIST main library in Chromeleon matched the mass spectral profiles to identify the aroma compounds, and then recorded molecular weights and retention times. For hierarchical clustering, peak areas were normalized per compound using $\log_2$(peak area/median of peak areas of all samples) *(van Rijswijck et al., 2017)*. Statistical Package for Social Science (SPSS) version 21 and Multi Experiment View (MEV) 4.9 software were used to statistically analyse the data and generate heat map clusters.

Results and discussion

The pH of the porridge inoculated with most of the single strain cultures and combinations of strains hardly dropped but stayed at pH around 6, indicating poor fermentation performance. Only with two single strain cultures (*Streptococcus thermophilus* and *Weissella confusa)* and particular strain combinations *(Lb-Lc-Le-St-We*, *Ac-Lb-Le-St-We*, *Ac-Lb-Lc-Le-St-We)*, the pH of the porridge dropped indicating fermentation activity (Figure 2).

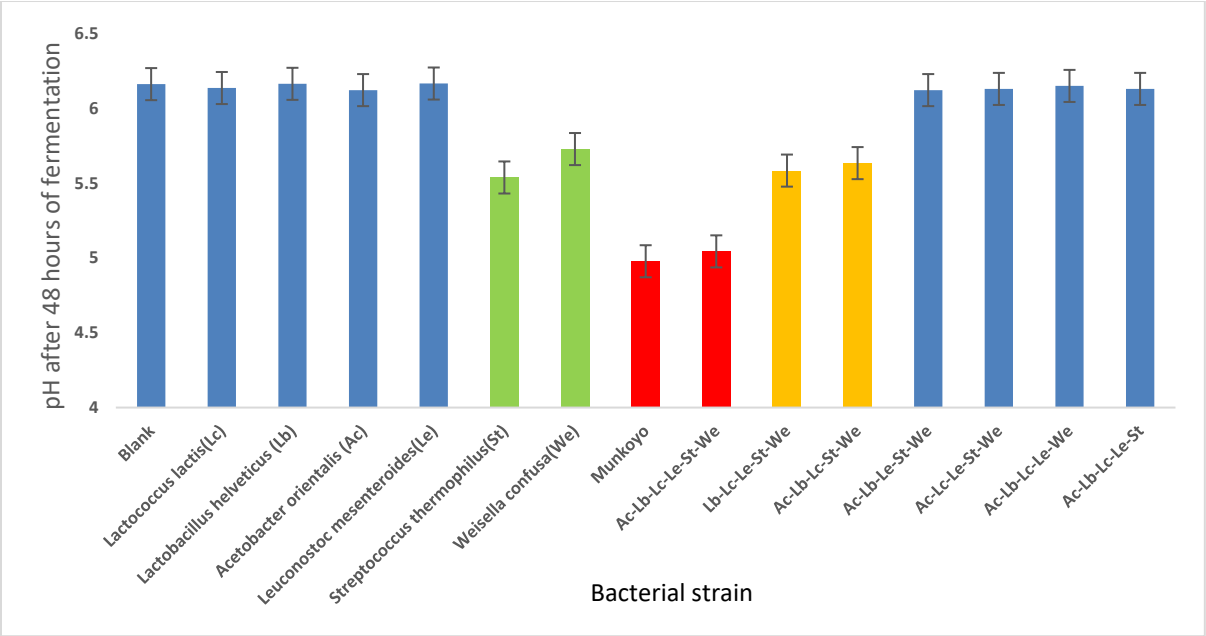

Figure 2. Final pH of Munkoyo after 48 hours of incubation upon inoculation with single bacterial strains Lc, Lb, Ac, Le, St and We and combinations of strains including a mixture of all six strains and 6 mixtures with a single strain omission. As a positive control Munkoyo was made with non-sterilised *Rhynchosia* root material. The error bars show standard deviation done in three replicates.

The absence of a pH drop in most of the single strain cultures and in some of the mixed strain cultures indicates limited bacterial growth and metabolism. Paired sample tests indicated no significant difference in the pH range after incubation between single strains and some combinations of strains with a p-value and standard deviation (P = 0.46 ±0.59). However, the pH of the positive control, i.e. the Munkoyo as traditionally prepared, and the combination with all single strains (Ac-Lb-Lc-Le-St-We) was significantly different with pH values lower than 6. The results show that a mixture of all six single strain bacteria (Ac-Lb-Lc-Le-St-We) provides a drop of the pH that resembles that of a fermentation process of traditional Munkoyo. Thus, the acidification of spontaneously fermented Munkoyo can be mimicked using a consortium of single strains of the species *A. orientalis*, *Lb. helveticus*, *L. lactis*, *Lc. mesenteroides*, *S. thermophilus* and *W. confusa*. This has also been observed in the spontaneous fermentation of maize bran in which the characterization of microbiota involved in natural fermentation process allowed the selection of specific biotypes with appropriate metabolic and enzymatic activities to conduct "tailored" fermentation processes and improve brans *(Decimo et al., 2017).*

In this study a total of 51 different aroma compounds were identified in Munkoyo either made with pure cultures or with different bacterial consortia as starter cultures or by spontaneous fermentation. Volatile compounds combined over all treatments consisted of 27.4% esters, 25.5% alkanes, 17.6% alkenes, 11.8% aldehydes, 9.8% organic acids, 4.0% aromatic alcohols and 7.8% alcohols as shown in Table 1. Similar groups of aroma compounds have been identified in fermented millet and sorghum beverages like Obushera of Uganda with complex mixtures that contained organic acids, aldehydes, ketones, alcohols and esters *(Mukisa et al., 2012)*. Aroma compounds that have been detected in Togwa, a cereal-based spontaneously fermented product in Tanzania include acetaldehyde, 2-methyl-propanal, 2-methyl-butanal, 3-methyl-butanal, ethanol, 2-methyl 1- propane, 2-methyl 1-butanol, 3-methyl 1 butanol, diacetyl and acetoin *(Mugula et al., 2003)*. Kenkey is another fermented maize beverage produced in Ghana and is prepared by spontaneous fermentation of a smooth dough prepared from milled, soaked maize grain and was found to contain the following aroma compounds: that include 21 carbonyls (22.1%), 19 alcohols (20%), 17 esters (17.9%), 12 acids (12.6%), a furan (1.1%), 2 phenolic compounds (2.1%), alkene (1.1%) and 4 unidentified (4.2%) *(Annan et al., 2003)*. Like many of the mentioned cereal-based beverages in Africa, Munkoyo also contains a wide range of aroma compounds such as esters, organic acids, aromatic alcohols, aldehydes, alkenes, alkane and alcohols (see Table 1).

Table 1. Fifty one aroma compounds identified in Munkoyo arranged in 7 groups of volatile compounds with percentages based on chemical identification and their groups.

Esters (27.4%)	**Alkanes (23.5%)**
Ethyl acetate	hexane
1-butanol,3-methyl-acetate	3,7-dimethylundecane
butanoic acid, 2-methyl- ethyl ester	2,4,5-trimethyl-1,3-dioxolane
pentanoic acid, ethyl ester	Isooctane
ammonium acetate	n-tetradecane
decanoic acid, ethyl ester	2,7,10-trimethyldodecane
2,3-butanediodiacetate	pentadecane
octanoic acid ethyl ester	2,3,5-trimethylhexane
propanoic acid, 2-hydroxy-ethyl ester	
heptanoic acid ethyl ester	
ethanedioic acid,bis(trimethylsilyl) ester	2,6,11,15-tetramethyl hexadecane
bis[2-(trimethylsilyl] malonate	hexadecane
2-furanmethanol, acetate	2,6,11-trimethyldodecane
ethyl nonanoate	tetradecane
Organic acids (9.8%)	**Alkenes (15.7%)**
hexanoic acid	benzene
n-hexadecanoic acid	ethylbenzene
acetic acid	(5Z)-2,6,10-trimethyl-1,5,9-undecatriene
butanoic acid, 3-methyl	naphtalene
2-methyl-propanoic acid	o-xylene
Aldehydes (11.8%)	1,4-dimethyl-4-vinylcyclohexen
2,3-butanedione	2-ethyl-1,4-dimethyl benzene
hexanal	benzene, 1-ethyl-2-4-dimethyl
2-heptanone	**Alcohols (7.8%)**
3,4-dimethyl-2-hexanone	ethanol
2-octanone	1-Pentanol
2-butanone, 3-hydroxy	p-menth-1-en-8-ol
Aromatic Alcohol (4.0%)	ethylhexanol
phenol	
phenol-4-ethyl	

A hierarchical cluster analysis of the aroma compounds of the differently produced Munkoyo samples indicates three important clusters. One cluster with the product made with single strain cultures of *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Weisella* plus most of the multiple strain mixtures with one omitted species [a], a second cluster containing the washing water control and *Lactococcus* cluster [b] and finally a cluster of Munkoyo samples, multiple strains bacteria*(Ac-Lc-Le-St-We*, *Ac-Lb-Le-St-We*, *Ac-Lb-Lc-Le-St-We)*, and *Acetobacter* as the only single strain bacteria cluster [c] shown in (Figure 3).

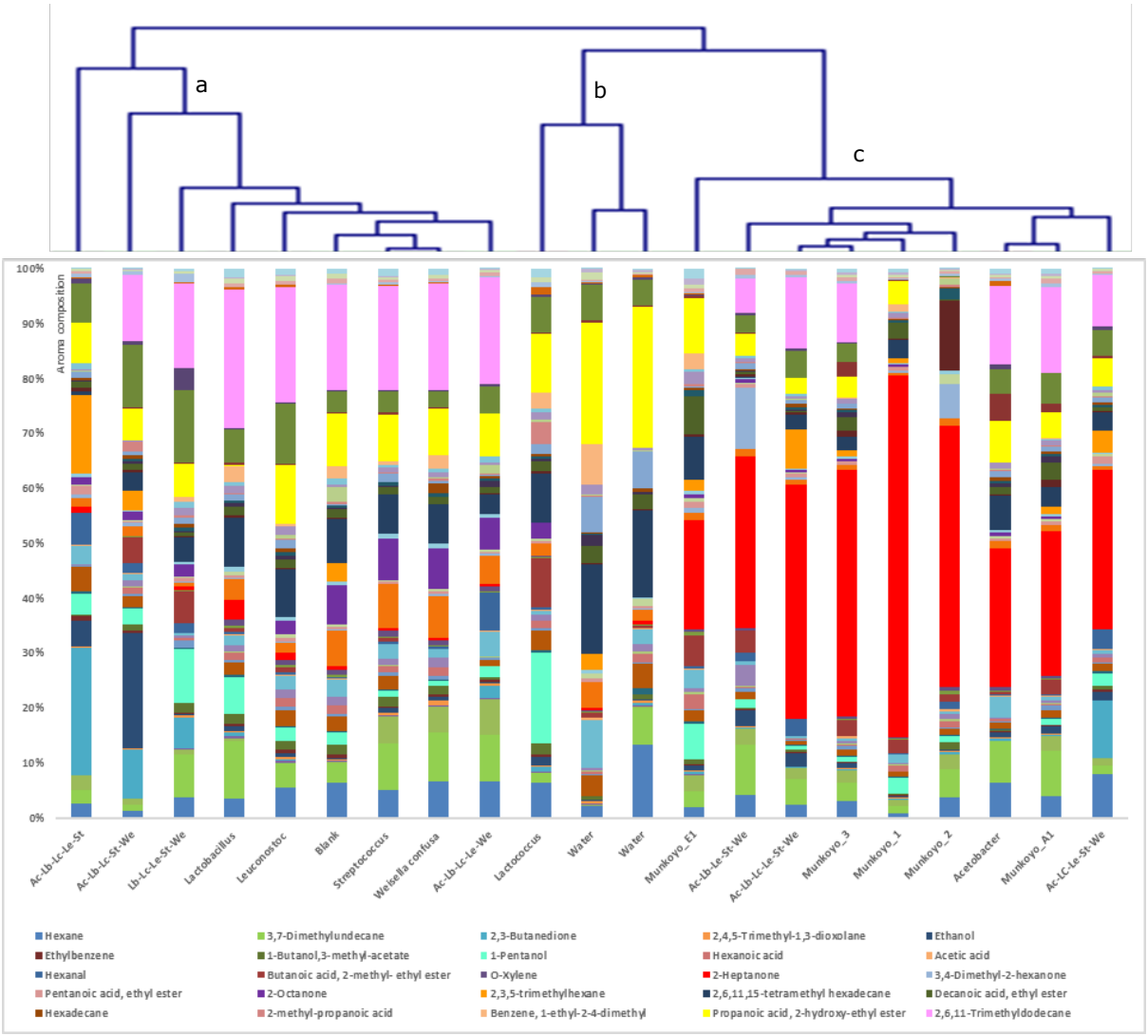

Figure. 3. Hierarchical cluster analysis of aroma compounds composition. Single strain bacteria and some multiple strain bacteria[a] clustered together with 2,6,11,Trimethyldodecane being dominant whilst Munkoyo and mostly multiple strain bacteria[c] clustered together with 2- heptanone as the most abundant aroma compound

Cluster [a] is characterized by a wide range of aroma compounds with 2,6,11 trimethyldodecane as the most abundant compound. Cluster [c] is characterised by the presence of a relatively high abundance of 2-heptanone. Munkoyo_3 had a perfect match with the multiple strain starter containing all six bacteria (*Ac-Lb-Lc-Le-St-We*) suggesting a potential starter culture of Munkoyo.

Thermal treatment is also known to produce a variety of Maillard reactions. The major volatile flavour compounds produced by Maillard reactions are heterocyclic compounds like thiophenes, thiazoles, pyrazines, thiazoles, pyrazines, pyrroles, imidazoles and pyridines *(Shibamoto, 1989)*. In

this study, Maillard reactions during cooking of Munkoyo may not have occurred, considering that none of the mentioned aroma compounds were detected.

Attempts to assess the contribution of LAB in the production of volatile organic compounds in traditional African cereal fermented products have been undertaken. For example, the individual and interactive effects of different LAB using single strain and mixed strain starter cultures containing *Weissella confusa, Lactobacillus plantarum, Lactococcus lactis* and *Lactobacillus fermentum* in the flavour profile of a traditional cereal-based fermented Ugandan beverage (Obushera) have been studied *(Mukisa et al., 2017)* and this showed that selection of suitable LAB strains may improve the flavour of malt based beverages *(Nsogning Dongmo et al., 2017)*. However, selection of the appropriate mix of bacteria for a starter culture involves evaluation of candidate strains for their viability, survival in the product, ability to utilize the major available carbohydrate substrates that contribute to the microbial growth as well as determination of the organoleptic, technological, nutritional and health advantages of the fermenting microbes *(Leroy & De Vuyst, 2004)*. In this research, the presence of *S. thermophilus* and *W.confusa* could be linked to high rates of acidification compared to other single strain cultures. Moreover, a mixture of strains containing all six bacterial species (*Ac-Lb-Lc-Le-St-We*) produced a profile of aroma compounds that was most comparable to that of Munkoyo made by spontaneous fermentation, indicating that a mixed culture consisting of these six strains of bacteria can serve as a starter culture in the production of Munkoyo.

Conclusion

The mixture of aroma compounds found in Munkoyo included 27.4% esters, 23.5% alkanes, 15.7% alkenes, 11.8% aldehydes, 9.8% organic acids, 4.0% aromatic alcohols and 7.8% alcohols. This study further revealed that single strain cultures of *S. thermophilus* and *W. Confuse* were able to ferment cereal porridge to pH values between 5.5 and 5.7. Moreover, the aroma compounds produced by a mixed culture containing six species of bacteria namely *A. orientalis, Lb. helveticus*, *L. lactis*, *Lc. mesenteroides*, *S. thermophilus* and *W. confusa* showed a close resemblance to the aroma profile of traditionally produced Munkoyo. This suggests that developing a starter culture of Munkoyo

may require a mixed culture with strains of at least these six species of bacteria which most likely interact to produce acids and aroma compounds that drive the production of a fermented product that closely resembles the spontaneously fermented Munkoyo. More research is required to determine how texture, sensorial properties, and shelf life of the product fermented with the multiple strain starter culture resemble those characteristics of traditional Munkoyo.

CHAPTER – FIVE

The role of *Rhynchosia* roots in the production of Munkoyo, a cereal-based fermented beverage

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Abstract

Munkoyo is a cereal-based fermented beverage mostly produced in Zambia and Democratic Republic of Congo (DRC). It utilizes *Rhynchosia* roots as an inoculum to provide amylases to degrade gelatinized starch into fermentable sugars. The available fermentable sugars are converted into organic acids, predominantly by lactic acid bacteria, to deliver the desirable sensorial attributes to the beverage. Fermentation of Munkoyo is a spontaneous process, which does not require a starter culture to initiate fermentation. The addition of *Rhynchosia* roots to the sterile porridge after cooking and cooling down to ambient temperature, makes the roots a potential source of lactic acid bacteria that ferment the beverage. This research addressed the role of *Rhynchosia* roots as a source of lactic acid bacteria, comparing millet malt and wheat flour as alternative sources of inoculum. We found that at the start of a fermentation cycle, microbial communities derived from *Rhynchosia* root were dominated by non-lactic acid bacteria such as Propionibacteriaceae, whilst at the end of fermentation the use of *Rhynchosia* roots resulted in domination of lactic acid bacteria (Lactobacillaceae, Enterobacteriaceae, Leuconostocaceae and Streptococcaceae). This suggests that *Rhynchosia* roots are as the source of lactic acid bacteria that proliferate during the spontaneous fermentation of Munkoyo. The use of both millet malt and wheat flour resulted in a particular viscosity, pH, TTA and bacterial community composition dominated by lactic acid bacteria similar to what is obtained when *Rhynchosia* roots are used, indicating that millet malt and wheat flour can both act as alternatives to *Rhynchosia* roots as source of amylolytic enzymes and bacteria for successful fermentation of Munkoyo.

Introduction

Many fermented beverages exist that are based on maize and other cereals rich in starch. Production of fermented cereal-based beverages relies on cooking a mixture of unmalted cereal meal and water to allow starch to gelatinize. Next, fermentable sugars are generated through hydrolysis of starch by the activity of amylolytic enzymes. Starch is the main carbohydrate in most cereals and hydrolysis is an enzyme-catalyzed process that breaks down long chains of starch into simpler, fermentable sugars. In turn, these fermentable sugars are converted into organic acids, predominantly by lactic acid bacteria, to generate a sour beverage with a characteristic taste, flavour, acidity, digestibility and texture *(Zvauya et al., 1997)*.

The origin of the amylolytic enzymes required for starch degradation varies. Many processing methods rely on the addition of malted sorghum or millet, which contain large amounts of amylases. For instance, to produce Togwa in Tanzania, malted millet is added to gelatinized starch *(Mugula et al., 2003)*, for Maheu in Zimbabwe either malted sorghum or millet is used *(Gadaga et al., 1999)* and finally, malted millet is used for Kwete in Uganda *(Muyanja & Namugumya, 2009)*. Malting of sorghum and millet provides α and β-amylases, which break gelatinized starch from the unmalted maize into fermentable sugars *(Saxena & Singh, 2011, Subbarao et al., 1998)*. As an exception, Munkoyo relies on *Rhynchosia* roots to provide the amylolytic enzymes *(Zulu et al., 1997)*. Munkoyo is a cereal-based traditional fermented beverage widely consumed in Zambia and the Democratic Republic of Congo (DRC) *(Foma et al., 2012, Foma et al., 2013)*. The fermentable sugars produced by the enzyme activity of the *Rhynchosia* roots consist of maltose (80% of all sugars), maltotrioses (17%) and glucose (3%) *(Zulu et al., 1997)*. The availability of these fermentable sugars yields a less viscous, free-flowing beverage with a good mouth feel and sweet taste. Mulkay *et al.* (1995) reported the presence of α and β-amylases in root extract of *Rhynchosia insignis* and *Eminia holubii*, which produced starch hydrolysates up to concentrations of 65-75% (w/w) *(Mulkay et al., 1995)*. Apart from the supply of starch degrading enzymes, *Rhynchosia* roots also contribute the yellow colour of Munkoyo beverage, explained by the flavonoids extracted from the added root material during

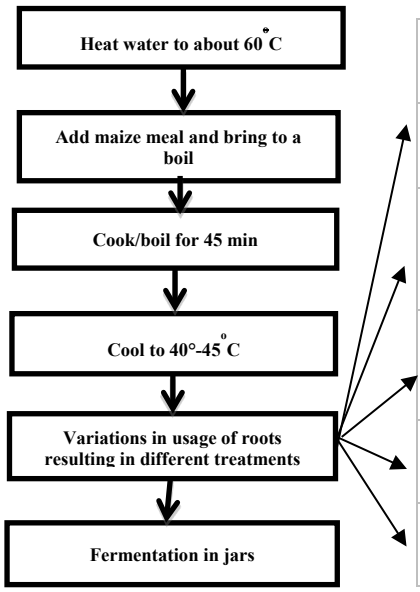
fermentation, although the final product after fermentation does not exhibit the same colour intensity because the colour fades as acidity increases *(Zulu et al., 1994).*

For the conversion of the fermentable sugars during processing, these traditional beverages rely on spontaneous fermentation, i.e. fermentation without the use of a defined mix of microbes (starter culture). To date it remains unclear the source of these microbes. The supply of microorganisms during spontaneous fermentation is perceived to be from fermenting utensils or raw materials *(Sahlin & Nair, 2012, Motlhanka et al., 2018)*. In addition, residues of the fermented product could provide bacterial inoculum during back slopping, for instance in the spontaneous fermentation of teff sourdoughs *(Harth et al., 2018).* The fermentation of Munkoyo does not depend on the microflora from the maize meal since this is thoroughly cooked before starting the fermentation process, nor does the fermentation depend on a back slopping process. While the bacterial communities responsible for Munkoyo fermentation have been identified *(Schoustra et al., 2013)*, the source of these bacteria remains hitherto unknown.

This research paper reports on a study to investigate *Rhynchosia* roots as a possible source of bacteria that are essential in the production of Munkoyo. We hypothesise that *Rhynchosia* roots are the source of bacteria since they are the only addition to the sterilized porridge after cooking, and this occurs prior to spontaneous fermentation at ambient temperature. We firstly compared the microbial community of *Rhynchosia* roots and the root washed with hydrogen peroxide. Secondly, we explored various alternatives to *Rhynchosia* roots as source of amylolytic enzymes and fermenting bacteria. This research is also motivated by the increasing scarcity of the *Rhynchosia* roots within people's habitations, which has contributed to occasional use of resembling roots that contain toxic substances. Thus, developing ways to replace the use of the *Rhynchosia* roots to supply the starch degrading enzymes and as possible source of necessary bacteria can curb the emerging problem of *Rhynchosia* root scarcity.

Materials and methods

Four different kinds of inoculum for the production of Munkoyo were studied, namely *Rhynchosia* root shreds, *Rhynchosia* root extract, wheat flour and millet malt. They were obtained from processors who offered the beverage for sale on the market. As common practice, roots are dug out from the *Rhynchosia* shrubs, dried and finally shredded. The general method to process Munkoyo and the variations in usage of the inoculum resulting in different treatments are shown in Figure 1. Three independent replicates were performed in clean jars with no previous fermentation.



Variation (code)	Description
Blank (B)	No addition of any root or extract; thus no addition of any microbial inoculum nor any amylolytic enzymes
Washed roots (washed-R) and extract (washed-E)	Roots that were washed using hydrogen peroxide removing all bacteria from the roots and their extract, not destroying any amylolytic enzymes.
***Rhynchosia* roots (*Rhynchosia*-R) and their extract (*Rhynchosia*-E)**	Positive control; normal common use of root inoculum
Flour	Alternative to *Rhynchosia* roots as source of microbial inoculum and amylolytic enzymes
Millet	Alternative to *Rhynchosia* roots as source of microbial inoculum and amylolytic enzymes

Figure 1. General processing methods of Munkoyo (left) and variations in inoculation treatments (right) to determine the source of bacteria in Munkoyo.

Twenty grams of shredded *Rhynchosia* roots were dipped in 100 ml of 70% (Vol/Vol) hydrogen peroxide for 60 sec followed by rinsing with running water. *Rhynchosia* root extract was obtained by soaking either 20 g of *Rhynchosia* roots or roots washed with 70% (Vol/Vol) hydrogen peroxide in 100 ml of distilled water for 20 min and subsequent sieving. In 1 L of gelatinized porridge in clean jars, 5 g of shredded *Rhynchosia* roots, wheat flour, millet malt or 10 ml of root extract was added and mixed thoroughly for the enzymes to be in contact with the gelatinized starch.

The pH was recorded using a pH meter (Hanna 212 model, Sigma-Aldrich) after every 12 h of fermentation for a total period of 48 h. Then 15 ml of the sample was collected for titratable acidity and viscosity measurements. Titratable acidity was determined by titrating 10 cm^3 of the sample against sodium hydroxide with phenolphthalein as indicator. Viscosity was measured by pouring 2 ml - 4 ml of hydrolysed porridge on the centre of concentric rings. The porridge with high viscosity spread uniformly outwards of the rings and the porridge with low viscosity haphazardly spread across the rings.

Samples were analysed for bacterial community composition after 48 h of fermentation. Bacterial community profiles were generated using non-culture based methods using 16S amplicon sequencing of full bacterial community DNA extracts. DNA extraction was done by spinning down 1 ml of Munkoyo sample at high speed after which the supernatant was discarded. Next, 500 µl TESL, 10 µl mutanolysin solution and 100 µl lysozyme solution were added to the pellet, followed by incubation at 37°C for 60 min with slight shaking. Then 500 µl GES reagent was added, followed by cooling on ice for 5 min. Subsequently 250 µl of cold ammonium acetate solution was mixed gently, followed by keeping the mixture on ice for 10 min before spinning and collecting the supernatant. The supernatant was purified with chloroform-2-pentanol by mixing 1:1, spinning down at 12,000 rpm and collecting the supernatant. DNA was precipitated by adding 0.1 volume of 3 M sodium acetate, followed by 2.5 volumes of 100% ethanol, and storage at -20°C overnight. Next the mixture was spun for 20 min at 12,000 rpm at 4°C, after which the supernatant was removed. Finally, the DNA pellet was washed out by adding 1 ml of cold 70% ethanol and spinning for 10 min at 12,000 rpm at 4°C. After removal of the supernatant, the DNA pellet was air dried for 10 min at room temperature, and dissolved in 10 mM Tris pH 7.5 *(Schoustra et al., 2013)*. Chimeric sequence checking, removal of noises from pre-cluster and taxonomic attribution were performed using standard parameters of QIIME (Quantitative Insights Into Microbial Ecology) software package, under MobXterm version 6. Applying the UCLUST method, sequences

presenting identity above 97% were the operational taxonomic units (OTUs) considered *(Neto et al., 2018)*.

Results

Physicochemical properties

The pH trajectories of all fermentations indicate that using the *Rhynchosia* roots, wheat flour and millet malt caused a pH drop to below 4 within 24 h. The blank (no roots added) and the roots washed with hydrogen peroxide did not result in the drop in pH, not even after 48 h (Figure 2).

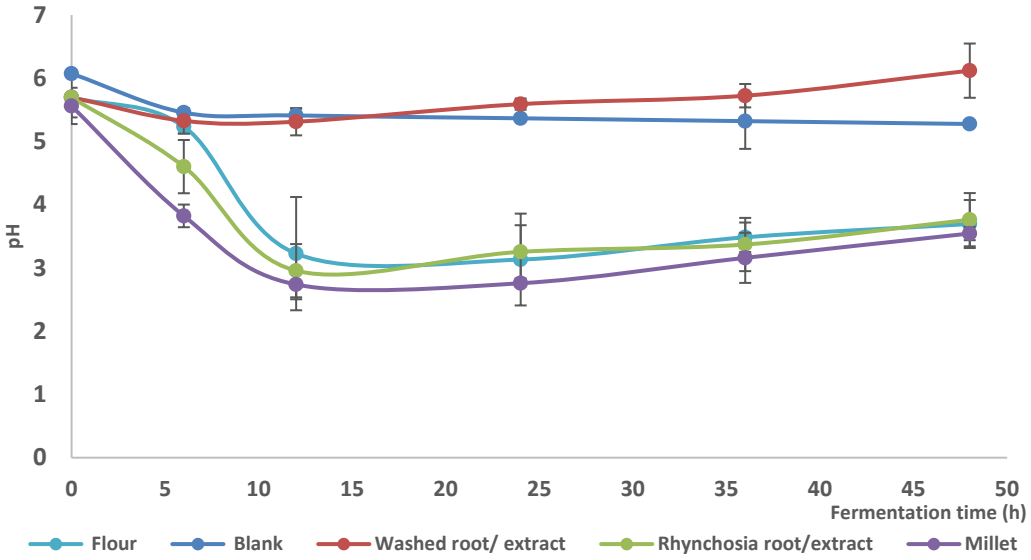


Figure 2. pH trajectory of maize fermentations following different inoculation treatments for the production of Munkoyo. Lines show averages of three independent replicates per treatment. Error bars show standard deviation.

Measurements of other physiochemical properties indicating titratable acidity, viscosity and their interpretation are shown in Table 1.

Table 1. Physicochemical parameters of Munkoyo prepared with different inoculum treatments Rhynchosia root (*Rhynchosia*-R or E), washed root with hydrogen peroxide (Washed-R or E), millet, flour and blank

Treatment	pH at 48 h	Titratable acidity	Viscosity	Interpretation
Blank	5.5	0.02	High	No acidification, no breakdown of starch
Washed root (Washed-R or E)	6.0	0.09	Low	No acidification, enzymatic breakdown of starch
***Rhynchosia* roots** (*Rhynchosia*-R or E)	3.8	0.21	Low	Acidification, enzymatic breakdown of starch
Flour	3.8	0.19	Low	Acidification, enzymatic breakdown of starch
Millet	3.5	0.34	Low	Acidification, enzymatic breakdown of starch

Bacterial community composition

The composition of the bacterial communities was determined at the beginning (t=0 h) and the end (t=48 h) of each fermentation. Results are shown in Figure 3a. Samples taken at t=0 h had more diverse bacterial communities than samples taken at t=48 h with many different bacterial genera of Propionibacteriaceae dominating. At (t=48 h), lactic acid bacteria (Lactobacillaceae, Enterobacteriaceae, Leuconostocaceae and Streptococcaceae) dominated the bacterial communities in samples that had experienced a significant drop in pH indicating bacterial growth of lactic acid bacteria. In samples where no drop in pH occurred (the blank and treatments washed with hydrogen peroxide) no changes in bacterial community composition at the level of genera occurred (Figure 3). Shannon diversity index, Effective Number of Species (ENS) and Evenness were calculated based on the observed number of unique species per sample. Shannon diversity index (H) mathematically expressed as $H = -\sum P_i \ln P_i$ measures diversity of species in a community. The higher the Shannon index the more diverse the species are in a community. ENS on the other hand represents the true diversity of the species in a community, mathematically expressed as $ENS = e^H$, whilst Evenness measures relative abundance of species in a community *(Grabchak et al., 2017)*.

Samples at t=0 h had a relatively diverse bacteria community with a Shannon index going up to 2 and an Effective Number of Species (ENS) around 9, which dropped to around 0.92 and 2.51, respectively, as acidity increased at t=48 h in the case of *Rhynchosia* root samples that showed normal acidification of Munkoyo (Figure 3b). Evenness remained rather constant between 0.5 and 0.6 over all communities sampled at t=0 and t=48 h.

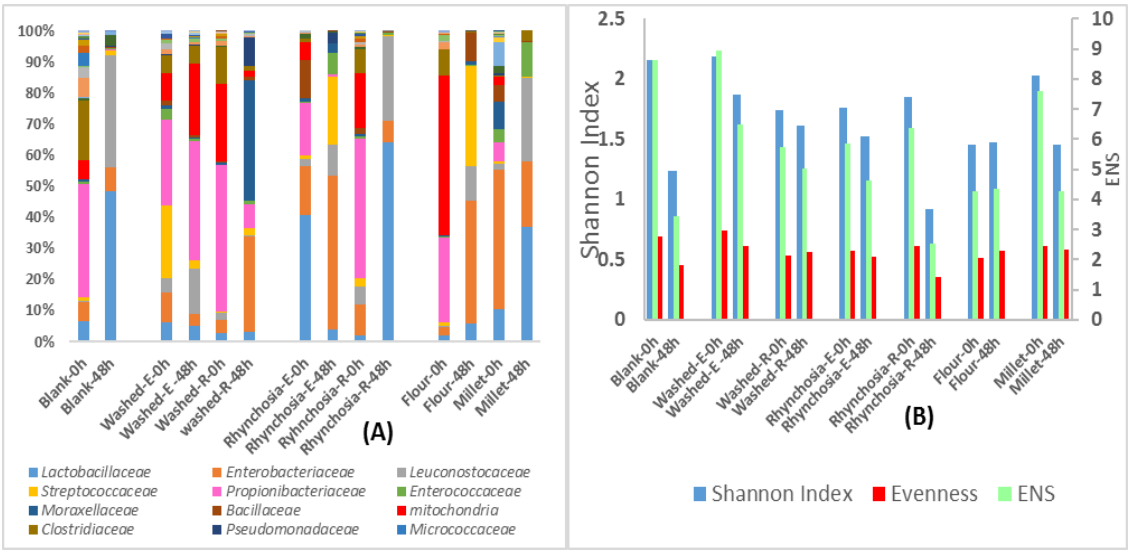


Figure 3. Panel (A) shows the microbial community composition of samples taken at the start (0h) and at the end (48h) of each fermentation treatment. After 48 h, the microbial communities in the washed treatment (Washed-R or E) did not change much from the start of fermentation and are characterized by non-lactic acid bacteria, namely Propionibacteriaceae, with traces of lactic acid bacteria, whilst *Rhynchosia* root treatment (*Rhynchosia*-R or E) resulted in a microbial community dominated by lactic acid bacteria (Lactobacillaceae, Enterobacteriaceae, Leuconostocaceae, Streptococcaceae). Panel (B) shows three diversity indices. Results show that microbial communities obtained after the washed root treatment had a higher Effective Number of Species (ENS) than *Rhynchosia* root treatment.

Discussion

We firstly established that *Rhynchosia* roots are a source of fermenting bacteria. When using roots or extract washed with hydrogen peroxide, no changes in pH or titratable acidity were observed between the start and the end of the fermentation trial, implying no growth of lactic acid bacteria. In addition, the profiling of bacterial communities obtained after 48 h did also not reveal a high abundance of lactic acid bacteria. In contrast, *Rhynchosia* roots as in the common practice, resulted in a sour product with common Munkoyo viscosity, pH, and titratable acidity, as well as bacterial communities dominated by lactic acid bacteria as indicated in previous studies *(Schoustra et al., 2013).* The use of both millet malt and wheat flour resulted in a viscosity, pH and titratable acidity that is customary for Munkoyo, and yielded a bacterial community composition in the final product that

was dominated by lactic acid bacteria. This suggests that millet malt as well as wheat flour can act as alternatives to *Rhynchosia* roots as inoculum of amylolytic enzymes and bacteria for successful Munkoyo fermentation.

When using roots washed with hydrogen peroxide or their extract, the viscosity of the maize porridge decreased to levels normal for Munkoyo, confirming that enzymes are indeed originating from the roots, and not the bacteria. Changes of viscosity in gelatinized starch by the action of amylolytic enzymes determine the rate of liquefaction. Therefore, the higher level of amylases correspond to faster liquefaction processes that affect rheology and viscosity of the final product *(Goode et al., 2005)*.

Bacterial profiles of the communities at the start of the fermentation trials revealed a wide diversity of species that are naturally associated with the 'rhizosphere', the area of soil surrounding plant roots with soil's most reactions that play a pivotal role in soil microbe and plant interaction *(Bashir et al., 2016)*.The high abundance of Propionibacteriaceae suggests the abundance of these bacteria in the rhizosphere of *Rhynchosia* roots as has been identified in paddy soil *microbiomes (Hayashi & Furusaka, 1979)*. As shown by the abundance of Propionibacteriaceae in the blank treatment where no *Rhynchosia* roots were added, the maize as raw material may also harbour these bacteria as part of a diverse microbial community. Our method also detected mitochondria of plant origin that share the same 16S ribosomal genes as bacteria. The acidification was indicative of bacterial growth that shifted microbial community composition towards domination of lactic acid bacteria (Lactobacillaceae, Enterobacteriaceae, Leuconostocaceae, Streptococcaceae) similar to the previous work done on Munkoyo *(Schoustra et al., 2013, Zulu et al., 1997, Foma et al., 2012)*, and other fermented cereals and beverages *(Uzochukwu et al., 2016, Doyle et al., 2013)*. This suggests that *Rhynchosia* root contain the microflora important to initiate fermentation of Munkoyo and that the amylolytic enzymes degraded starch that generates a selective advantage for lactic acid bacteria that are able to outcompete other groups of bacteria.

While we did not analyse sufficient replicate samples to allow a formal comparison, our results suggest that the use of *Rhynchosia* roots and the alternatives millet malt and wheat flour do not result in major differences in bacterial community composition at the level of bacterial families in the final product. This is also true for the analysed physicochemical parameters (final pH, viscosity and titratable acidity), suggesting a need for further research into the suitability of these alternatives to ascertain the microbial composition that would produce good fermentation. This could help to come up with a starter culture that would produce Munkoyo with consistent sensorial attributes, free from pathogenic contamination and avoid the use of lookalikes of *Rhynchosia* roots, which occasionally are implicated in intoxication of the beverage.

CHAPTER – SIX

General discussion

Introduction

The aim of this thesis project was to get more understanding of (i) the common processing steps involved in making Munkoyo and (ii) the microorganisms involved in driving the fermentation process. Furthermore, the Munkoyo associated aroma compounds were investigated and finally the role of *Rhynchosia* roots as a potential source of lactic acid bacteria driving the spontaneous fermentation was assessed. Traditional cereal-based fermented beverages are an important part of diet and livelihoods in Africa. Munkoyo, a cereal-based, spontaneously fermented beverage in Zambia is such a product. Spontaneous fermentation depends on microorganisms from the environment or the normal microflora of the substrate to initiate the process (*Capozzi et al., 2017*). This implies that a diverse microbial community, consisting of various species, is involved in the fermentation process, which ultimately leads to variations in product quality, taste and aroma. The low pH of Munkoyo ensures reduced risk of spoilage and outgrowth of pathogenic bacteria. For that reason the beverage is recommended for children, besides, it provides energy and its non-alcoholic.

Results from the survey and microbial composition of Munkoyo

A survey conducted in four locations of the same agro-ecological zone (namely in Lusaka, Chongwe, Chibombo and Mumbwa) showed that Munkoyo is largely consumed as an energy drink in rural areas and as a social drink in urban areas where people have alternative foods to choose from. The fact that Munkoyo is consumed by the entire population, including women and children, suggests that Munkoyo can be used as a means to provide energy and nutrients. It should thus be encouraged to hygienically process and sell Munkoyo at local markets to generate income for the livelihood of indigenous processors.

Analysis of the microbial composition of Munkoyo indicated that lactic acid bacteria are the most abundant bacteria, responsible for the spontaneous fermentation of the beverage, which is in line with research on similar cereal-based beverages *(Embashu et al., 2013, Mugula et al., 2003, Muyanja & Namugumya, 2009, Schoustra et al., 2013).* The activity of lactic acid bacteria in the beverage

produces lactic acid that acts as preservative thereby increasing the shelf life of the product *(Achi & Asamudo, 2018),* which is especially relevant in rural areas due to lack of refrigeration. Apart from producing lactic acid, lactic acid bacteria potentially deliver probiotic effects upon ingestion of fermented foods. By definition probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host *(Xiao et al., 2014).* This includes bacteria that have beneficial effects on the gastrointestinal tract of the host. In East Africa, a dried probiotic starter culture containing *Streptococcus thermophilus* and *Lactobacillus rhamnosus* has been used to produce a probiotic cereal-based product with a long shelf life and good consumer acceptance *(Salmeron et al., 2015).* In West Africa, a starter culture containing *Lactobacillus* strains was used to produce improved Ogi that exhibited antimicrobial properties against diarrhoeagenic bacteria *(Achi & Asamudo, 2018)*. This is because most probiotics fall in the group of lactic acid-producing bacteria that exert a strong antagonistic activity against many pathogenic microorganisms and show particular potential as a practical vehicle to provide probiotic strains to people with compromised health *(Marco et al., 2017).* They are mostly consumed in the form of fermented milk, yogurt and other fermented products. The beneficial effects of probiotics include (i) improving the health of the intestinal tract, (ii) enhancing the immune system and bioavailability of nutrients, (iii) reducing symptoms of lactose intolerance, and (iv) reducing the risk of certain cancers *(Parvez et al., 2006).*

Many cereal-based beverages are dominated by lactic acid bacteria such as *Lactobacillus*, *Lactococcus* and *Leuconostoc.* *Lactobacillus* is usually the dominant genus with *L. fermentum*, *L. plantarum* and *L.delbrueckii* as important species. For Munkoyo, the presence of *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus brevis*, *Bacillus licheniformis* and *Weissella confusa* was confirmed *(Schoustra et al., 2013, Zulu et al., 1997).* This microbial community highlights the potential of Munkoyo to carry probiotics with possible antimicrobial properties against diseases like diarrhoea. There is also need to optimise the processing methods of making Munkoyo and its bacterial community composition to enhance the growth of lactic acid bacteria with probiotic properties.

Effects of different processing methods on the bacterial composition

The processing of Munkoyo is generally a biochemical conversion of maize meal as raw material by enzymes and microbes to produce a fermented product containing lactic acid, aroma compounds and nutrients (Figure 1). The final product has sensory attributes that varies from one processing method to the other. Specific differences in the processing of Munkoyo as a beverage exist among processors in different agro-ecological regions in Zambia.

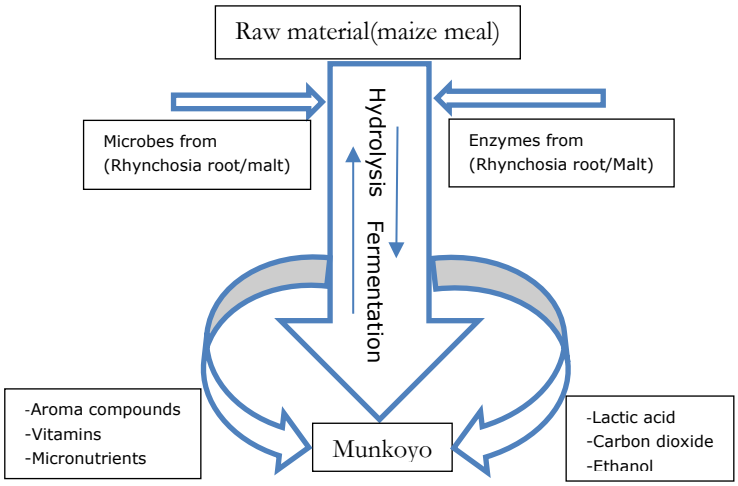


Figure 1. Biochemical conversion of maize meal into a Munkoyo by enzymes and microbes

In the Northern part of Zambia, around Kitwe, cooking of Munkoyo is extended to more than one hour to induce the generation of desired flavours by Maillard reactions such as pyrazines associated with roasting, cooking, or toasted baked cereals *(van Boekel, 2006)*. In the Southern part of Zambia, in Choma, maize grits are used instead of maize meal as raw material which gives it a lot of suspended starch from the grits. Furthermore, in this region root extract is used as an inoculum rather than the root itself. Here the beverage is given a special name, 'Chibwantu'. In the Eastern part of Zambia, in Nyimba, and most urban areas, maize meal is used as starting material and *Rhynchosia* roots are immersed in the porridge as an inoculum for at least a period of 24 h, in practice referred to as overnight incubation.

The composition of the microbial community that resulted from the three different processing methods (see Chapter 3), revealed that variations in processing methods significantly contribute to the variation found in microbial community composition (see Chapter 3). The measured microbial

community composition from the same processing method do not completely cluster together as monophyletic groups, and the processing location may also have significantly affected the microbial community. This could imply that environmental factors such as the bacterial community on *Rhynchosia* roots from a particular soil type and the utensils used in the processing have much influence on the bacterial consortium that ferments the product. This is supported by the fact that soils generally harbour diverse bacterial populations, whose composition is affected by soil characteristics such as pH *(Lauber et al., 2009)*.The fact that *Rhynchosia* roots used in the processing of Munkoyo originate from different agro-ecological zones with different soil types and pH, namely acidic soil in Kitwe, moderately acidic soil in Nyimba and relatively alkaline soil in Choma *(Chabala et al., 2014)*, might explain why the location where the product was produced significantly affected the microbial composition, as shown in Figure 2.

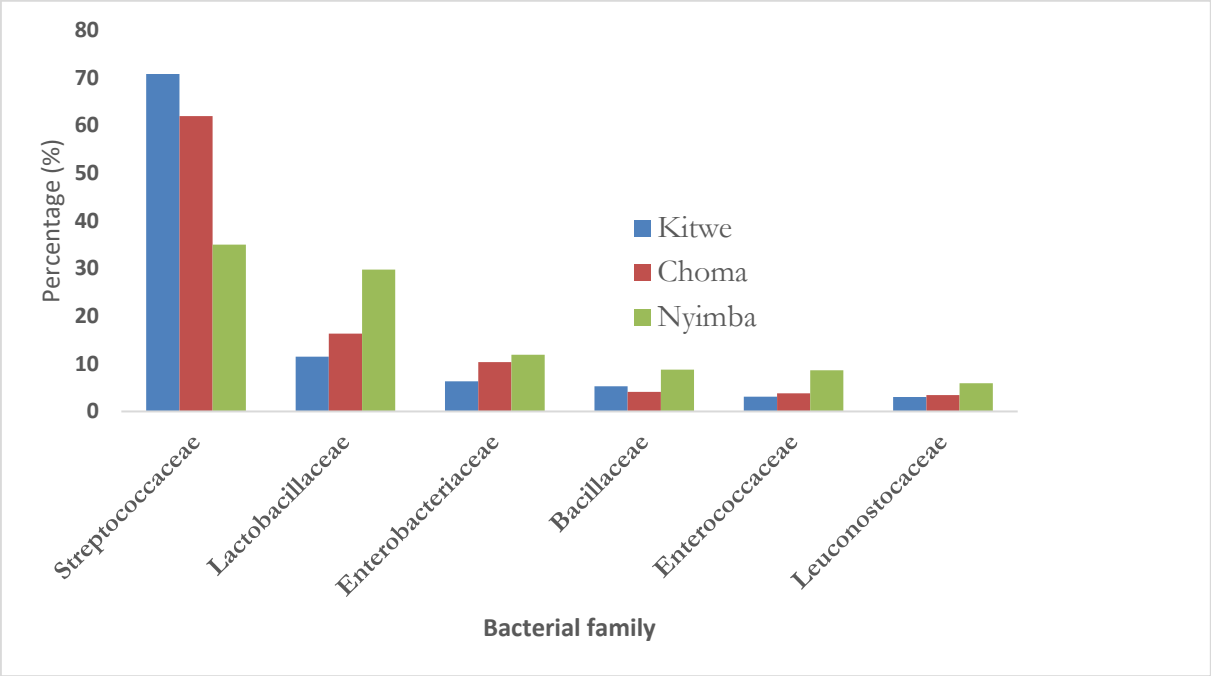


Figure 2. Microbial community composition of Munkoyo as related to the location of production, namely Choma, Kitwe and Nyimba

Interestingly, Streptococcaceae dominated in all the locations of this research. This finding is corroborated by other research that indicated that *Streptococcus sanguinis*, is the most dominant *Streptococcus* species in certain soils *(Gledhill & Casida, 1969)*, besides *Streptococcus* is among the most

common LAB genus associated with cereal fermentation *(Tamang et al., 2016)*. The microbial soil community is also known to be impacted by management practices and soil organic matter content. These aspects are main differentiating features between agro-ecological zones (*García-Orenes et al., 2013*). For example, soils around Kitwe have a low pH because of high rainfall that leaches the soil, compelling different soil management practices than in other regions.

Aroma compounds in Munkoyo

Fermentation of many traditional cereal-based foods is normally carried out through spontaneous (natural) fermentation involving mixed cultures of bacteria. Aroma compounds produced during fermentation have many volatile and nonvolatile components, possessing diverse chemical and physicochemical properties *(Longo & Sanromán, 2005)*. The variable microbial community makes it difficult to predict the common aroma compounds in different Munkoyo beverages. This is also true for other spontaneous lactic acid fermentations of cereal substrate, which show that the products' aroma composition varies among processors (*Nsogning Dongmo et al., 2016*). This probably explains why there are variations in the sensory attributes of the Munkoyo batches made by different processors in different regions.

In this research, we compared the aroma compounds produced from pure single strains, mixed pure strains and Munkoyo made from *Rhynchosia* roots. Not all single strain cultures were able to grow on the raw materials used to produce Munkoyo; only *Streptococcus thermophilus* and *Weissella confuse* as single pure cultures could grow and dropped the pH of the porridge indicating fermentation activity. The mixture of all six pure single strain cultures of *Acetobacter orientalis*, *Lactobacillus helveticus*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Streptococcus thermophilus* and *Weissella confuse* dropped the pH and also produced aroma compounds that resembled the aroma profile of traditional Munkoyo. Thus, the production of spontaneously fermented Munkoyo can be mimicked using a consortium of pure single strains of the species *A. orientalis*, *Lb. helveticus*, *L. lactis*, *Lc. mesenteroides*, *S. thermophilus* and *W. confusa*. This has also been done for an Ugandan cereal-based

beverage, called Bushera, made from sorghum flour, fermented sorghum or millet grains and water. To produce this beverage, a lactic acid starter culture of *Lactobacillus fermentum MINF99*, *Weissella confusa MINF8*, *Lactobacillus plantarum MINF277*, *Lactobacillus brevis MINF226* and *Lactobacillus paracasei* subsp. *paracasei MINF98* was used. It was found that *L. plantarum MINF227* and *W. confuse MINF8* were suitable lactic acid bacteria to produce rapid acidification and volatile flavour compounds characteristic for Bushera *(Muyanja et al., 2012)*. This suggests that specific strains of lactic acid from a consortium of bacteria may play a large role in the production of desirable sensory attributes in the beverage. However, as long as such knowledge is unavailable for spontaneous fermentation with microbial compositions that vary among processors, it is recommended to include all identified bacteria of the consortium.

Role of *Rhynchosia* roots

Munkoyo has a special position among the cereal-based fermented beverages as *Rhynchosia* root is used as an inoculum to provide amylases to degrade gelatinized starch into fermentable sugars. This degradation of starch is essential to allow fermentation, since most lactic acid bacteria are not capable of using starch as a carbon source. Many other similar beverages across Africa use cereal malts to provide amylases. The fermentable sugars released by amylases are converted into organic acids by lactic acid bacteria, thereby providing desirable sensorial attributes to the beverage. The gelatinization process requires boiling of the cereal porridge for over an hour, eliminating all bacteria, including those present in the raw material that could have initiated spontaneous fermentation. Thus, the addition of *Rhynchosia* root to gelatinized starch at ambient temperature may also be the source of lactic acid bacteria that initiate spontaneous fermentation. The results of this study show that the most important bacteria that make up the consortium derived from the *Rhynchosia* roots, wheat flour and millet malt after 48 h of fermentation were bacterial species belonging the families of Lactobacillaceae, Enterobacteriaceae, Leuconostocaceae, Streptococcaceae, Enterococcaceae and Bacillaceae, largely lactic acid bacteria. This is desired as it

has been observed in most spontaneous fermentation of most cereal-based beverage across Africa *(Mugula et al., 2003, Uzochukwu et al., 2016, Blandino et al., 2003)*. Munkoyo produced by *Rhynchosia* root at 48 h (as the most practiced way of making Munkoyo) show very low microbial diversity with Shannon index of 0.92 and equally low Effective Number of Species (ENS) of 2.51 (See Chapter 5). This is expected because the abundance of lactic acid bacteria tend to suppress the growth of other non-lactic acid bacteria especially pathogens that cannot survive in low pH *(Sahlin & Nair, 2012)*. The abundance of Propionibacteriaceae in all treatments at 0 h (Chapter 5), could be due to the abundance of these bacteria in the rhizosphere around the *Rhynchosia* root as has been identified in paddy soil microbiomes *(Hayashi & Furusaka, 1979)*. It should be recommended however, to plate the treatments to verify the assumption that Propionibacteriaceae may be the abundant bacteria in the rhizosphere around the *Rhynchosia* root.

Formalization of Munkoyo processing

The production of Munkoyo is presently an artisanal activity that is passed on from one generation to the next. Spontaneous (natural) fermentation is still the most simple and economical way of obtaining Munkoyo with the desired sensory properties and functional qualities *(Blandino et al., 2003)*. Fermentation is generally known to reduce anti-nutritional factors in cereal-based foods and increase the bioavailability of certain amino acids and B vitamins (*Achi & Asamudo, 2018*). Despite differences in processing methods, regional processing methods like those identified in chapter 3, should still be encouraged to promote consumption of what local people prefer and like as long as they meet the quality criteria and provide the benefits that accompany fermented foods.

On basis of the fact that lactic acid bacteria dominated the fermentation of Munkoyo, it can be recommended that consecutive batches of Munkoyo have to be processed in the same vessel to create an optimised consortium of lactic acid bacteria that continuously produces the same desirable sensory attributes over and over again. In the long run, the microbial composition in such a fermentation vessel is expected to stabilize. Analysis of such back-slopped microbial consortia

could then provide insight into what can be considered as a representative starter culture. Ensuring hygienic storage conditions for *Rhynchosia* roots on the part of traders is advocated to reduce contamination by pathogenic bacteria and variability of the microbial community. This includes avoiding bunching the roots together with other materials that may be contaminated by pathogens. Commercial processing of Munkoyo can be advantageous to increase consumption, especially in urban areas where people presently do not have access to the product. However, processors should be able to deal with the challenge of conforming to the sensory attributes desired in traditionally processed Munkoyo. It can thus be recommended that commercial processors build batch fermenters that would develop an optimised consortium of lactic acid bacteria much like what is done by local processors who repeatedly ferment the beverage in the same calabash. This might reduce the variability that is currently observed between commercial processors and traditional processors of the beverage, thereby encouraging regular consumption by both rural and peri-urban population.

Nutritional aspects of Munkoyo

Fermented cereal-based beverages might be classified as novel fermented foods because they are carriers of functional food compounds, such as dietary fibre, vitamins, minerals and antioxidants *(Kreisz et al., 2008)*. In general, cereals often contain anti-nutritional components such as phytates, protease inhibitors and polyphenols (*Reddy & Pierson, 1994*). These phytates form complexes with micronutrients such as Ca^{++}, Fe^{++}, Mg^{++}, Mn^{++}, and Zn^{++}, making them insoluble and unavailable for absorption in the intestinal tract of humans *(Bohn et al., 2008)*. Fermentation by lactic acid bacteria not only preserves cereal-based products but can also decrease anti-nutritional components of these cereals and increase the bioavailability of nutrients as observed in *Tchoukouotou* from maize, *Uji* from sorghum, *Ben-saalga* from pearl millet, and *Jnard* from finger millet (*Waters et al., 2015*). Apart from the inhibition of the proliferation of pathogenic bacteria due to the low pH,

the fermentation process further enhances food safety by reducing toxic components such as aflatoxins and cyanogenic glycosides, (*Giraffa, 2004*).

Vitamins are micronutrients that are essential for the metabolism of all living organisms. They are found as precursors of intracellular coenzymes that regulate vital biochemical reactions in the cell *(Capozzi et al., 2012).* However, humans are incapable of synthesizing some of these functional compounds like niacin (vitamin B3), folate (vitamin B11-B9), riboflavin (vitamin B2), and thiamine (vitamin B1) *(Stanton et al., 2005).* These vitamins are easily removed or destroyed during cooking or processing. As an alternative to chemical synthesis or fortification, fermentation with food grade lactic acid bacteria offers a more natural and economical opportunity to improve the nutrition value of cereal-based products and the development of novel functional foods (*Achi & Asamudo, 2018*). For instance, important lactic acid bacteria such as *Lactococcus lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* have the ability to synthesise folate (vitamin B11) in yogurt starter cultures *(Laiño et al., 2012)* and possibly non milk-based products such as cereals. The presence of similar lactic acid bacteria in spontaneous fermentation of Munkoyo might increase the levels of vitamins from the B group in Munkoyo. Thus, the development of novel functional cereal-based foods with enhanced vitamin content as a result of fermentation will not only increase their commercial and nutritional value but also abolish the need for chemical fortification of foods with vitamins.

Maize as a staple crop is usually processed into flour that is prepared into porridge for children and into a thick porridge, called Nshima in Zambia, as a principal energy giving dish in most Southern African countries, including Zambia. Munkoyo is another product from maize flour, consumed as a fermented drink at social gatherings and at household level. Despite the fact that maize is deficient in some essential amino acids like lysine, fermentation may be the most simple and economical way of improving its nutritional value, sensory properties and functional qualities (*Blandino et al., 2003*). The release of maize varieties that produce orange seeds has received great attention in Zambia because it contains many important vitamins except Vitamin B12 (*Nuss & Tanumihardjo, 2010*).

Processing of Munkoyo using orange maize is a step towards improved intake off at soluble vitamins and micronutrients that are not readily available in white maize flour. This is an area of research that warrants further study.

Conclusion

Maize is the most consumed and multipurpose crop in Southern African countries. A whole grain is a rich source of carbohydrate and fibre with moderate protein and micronutrients. Bioavailability of nutrients is influenced by levels of anti-nutritional factors, storage conditions, processing and cooking procedures. Fortification to improve limited levels of amino acids, B-Vitamins and trace minerals in maize is highly encouraged but rather expensive. Fermentation on the other hand is the ancient and cheapest method of preserving food raw material. Fermentation of cereals in particular is expected to further improve the nutritional composition, bioavailability of most nutrients, digestibility, shelf life as well as sensory properties.

While spontaneous fermentation generally enhances the safety of foods due to a reduction in pH, and through detoxification, in some cases there are safety concerns related to bacterial pathogens associated with the raw material or unhygienic practices during processing. Further research directed at optimizing the processing methods and characterization of microorganisms in the production of maize-based fermented products (eg. Munkoyo) to improve safety and nutrient benefit is necessary. Further, effective ways of enhancing the nutritional quality of maize-based foods through blending with available nutrient rich foods to improve the bioavailability of essential nutrients through fermentation of different traditional processing methods in maize is recommended.

Recommendations

- Measurements of soil pH, organic matter and microbes can provide the link between the microbial community in the soil and the *Rhynchosia* root. It is recommended to analyze the

physicochemical properties and microbes of both the soil and *Rhynchosia* roots and then further compare the microbes found after spontaneous fermentation.

- Fermentation of the beverage in the same vessel with an optimized consortium of lactic acid bacteria is presently recommended as the most convenient and cheapest processing practice for the small-scale producers of the beverage.
- The link between specific microbial communities and the aroma profile responsible for the characteristic sensory attributes in Munkoyo is important. Thus the fact that there is a wide variation of microbial communities underlying Munkoyo fermentation may have consequences for reproducing the characteristic sensory attributes in Munkoyo. It is thus recommended to design processing techniques that will ensure the growth of microbial communities that produce consistent and desirable sensory attributes in Munkoyo.
- Cereals are known to contain anti-nutritional factors like phytates that make micronutrients such as the divalent cations Ca, Fe, Mg, Mn, and Zn unavailable for absorption in the intestinal tract of humans. Fermentation of cereals is known to reduce such anti-nutritional factors in cereals and lead to increased bioavailability of important minerals needed by the human body. It is thus recommended to encourage production and regular consumption of Munkoyo at household level to be able to access these micronutrients in Munkoyo as a result of fermentation.
- Occasionally there cases of intoxication after consuming Munkoyo due to use of toxic roots that look like *Rhynchosia* roots. There is need for research into identifying the species of toxic roots and then educate the collectors to avoid collecting roots that may lead to intoxication upon consumption of the beverage.

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Thesis summary

Fermentation is the oldest technology used in the production of many traditional cereal-based beverages across Africa. Munkoyo, a spontaneously fermented cereal-based beverage from Zambia and Democratic Republic of Congo is one of those beverages and the focal product studied in this thesis. It is consumed by large parts of the population, as an energy drink during travel or work on the field and is also consumed at social gatherings like wedding festivals and funerals. The processing of Munkoyo has to date not been standardized and greatly varies between different regions and processors. This may affect the quality and the safety of the beverage. Optimization of the processing methods and microbial composition of Munkoyo to attain good quality and a safe product can be a milestone leading to increased consumption of the beverage.

Like all fermented foods, processing of Munkoyo relies on microbial activity transforming raw materials (cereals such as maize, millet and sorghum) into a processed product. The thesis focused on understanding the art of making Munkoyo by surveying different processors and linked this to the microbial community composition of fermenting microbes and their functionality in producing aroma compounds. Further, we investigated whether the source of the fermenting bacteria could lie in the *Rhynchosia* roots that are added during processing.

Chapter 2 describes a survey using a semi structured questionnaire and focus group discussions in Lusaka, Chongwe, Chibombo and Mumbwa. The survey revealed that Munkoyo is consumed by the entire population, as an energy drink, during long hours of manual work, at social gatherings and is mainly processed at household level. Characterization of the bacterial communities of over 90 samples with 16S amplicon sequencing on DNA extracted from the entire bacterial community revealed six families of mainly lactic acid bacteria to dominate the bacterial communities. **Chapter 3** reports that there are principally three processing methods of making Munkoyo with reference to three different agro-ecological zones of the country. Microbial community composition is affected by processing method. **Chapter 4** describes a study where we used six single strain cultures

of bacteria commonly found as members of the microbial communities in Munkoyo and combinations of them to determine the most influential bacteria or groups of bacteria in the production of aroma compounds. We found that spontaneously fermented Munkoyo and Munkoyo made with the combination of all six species of bacteria had a similar composition of aroma compounds compared to the regular product implying that a complete mixture of microbes is required to potentially be used as starter culture for Munkoyo production. **Chapter 5** reports on a study addressing the question if the addition of *Rhynchosia* root during processing provides the source of lactic acid bacteria responsible for fermentation. The study showed that microbial communities present on the roots used to make Munkoyo are mainly non-lactic acid bacteria like Propionibacteriaceae. Lactic acid bacteria were present at relative abundances below ten percent. After fermentation, the resulting product was dominated by lactic acid bacteria (Lactobacillaceae, Enterobacteriaceae, Leuconostocaceae, Streptococcaceae), which is a normal microflora in most cereal-based beverages. This supports the idea that *Rhynchosia* root is a potential source of lactic acid bacteria in spontaneous fermentation of Munkoyo.

The work presented in this thesis highlights that spontaneous fermentation of Munkoyo possesses the challenge of product inconsistence from different processing methods. Further, the consequences and risks associated with contamination by pathogenic bacteria remains to be explored. Optimizing the processing methods and standardizing the microbial composition by administering starter culture can ultimately enhance quality and safety of the product to formalize production, increase consumption and contribute to food security.

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2019 October, Wageningen the Netherlands.

List of Publications

Mugode. L, Kaunda. A, Sikombe .T, **Phiri. S**, Mutale.R, Davis.C, Tanumihardjo.S, De moura F. (2014), Carotenoid retention of biofortified provitamin A maize *(Zea mays. L.)* after Zambian traditional methods of milling, cooking and storage. Journal of Agriculture and Food Chemistry 62(27), 6317-25

About the Author

Sydney was born in Lusaka, Zambia on the 10th of March 1974. He grew up in Lusaka where he attended his primary education at Ellensdale Primary School. He did his high school at Kafue Boys Secondary School. After finishing high school he trained as a Secondary School Teacher of Science and Mathematics. He was employed by Ministry of Education to teach Science and Mathematics at Lotus basic School in 1997.

Two years later he was admitted to the University of Zambia and Graduated with a Bachelors degree in Food Science and Technology (BFST) in June 2005. He was then transferred from Ministry of Education to Ministry of Agriculture at Zambia Agricultural Research Institute (ZARI) as a researcher where he is currently working.

1n 2012 he got AUSAID Scholarship to do a Master of Food Science and Technology at Curtin University of Technology. One year after returning from Australia he got a scholarship to pursue a PhD degree at Wageningen University themed '**Spontaneous fermentation of Munkoyo; a cereal-based beverage in Zambia**, a thesis defended on Tuesday the 15th October 2019.

Overview of training activities

Category A: Discipline specific activities

Courses	City	Year
Food and Biorefinery Enzymology	(VLAG) Wageningen	2015
Multivariate Analysis	(PE&RC) Wageningen	2015
Aroma Compounds	(VLAG) Compenhagen	2015
Food Fermentation	(VLAG) Wageningen	2016
Reaction Kinetic in Food Science	(VLAG) Wageningen	2016
Food Value Chain Research	(WASS) Wageningen	2017
Masterclass Applied Biocatalysis	(VLAG) Wageningen	2019

Category B: General courses

VLAG PhD week	(VLAG) Baarlo	2015
High Impact writing in Science	(WAIS) Wageningen	2015
Techniques of writing and presenting scientific paper	(WGS) Wageningen	2015
Bayesian Statistics	(PE&RC) Wageningen	2015
Introduction to R statistic Analysis	(PE&RC) Wageningen	2015
Entrepreneurship in and outside science	(WGS) Wageningen	2015

Category C: Optional courses

Preparation of research proposal	(FQD) Wageningen	2015
PhD study tour	Milan	2016
Weekly group meetings	(FQD) Wageningen	n.a.
PhD study tour	Sydney & Melbourne	2018

Colophon

The research of this thesis was carried out in Netherlands, Wageningen University and Research under (Food Quality and Design, Laboratory of Genetics and Food Microbiology) and in Zambia; University of Zambia under School of Agricultural Science in the Department of Food Science and Nutrition. This PhD thesis was a part of a project on Zambian traditional fermented foods (Munkoyo and Mabisi) funded by **NWO-WOTRO** under the Food and Business Global Challenges Programme **(W08.250.2013.108)**.