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Microbial succession in indigenous fermented cereal beverages of Lesotho

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Submitted in fulfilment of the requirements in respect of the Master's degree in MICROBIAL BIOTECHNOLOGY at the University of the Free State.

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I Bokang John Mahlomaholo hereby declare that this work, submitted for the Master's degree, at the University of the Free State, is my own original work and has not previously been submitted, for degree purposes or otherwise, to any other institution of higher learning. I further declare that all sources cited or quoted are indicated and acknowledged by means of a comprehensive list of references. Copyright hereby cedes to the University of the Free State.

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Dedications

I dedicate this thesis to my mother, Ntšabeng Amelia Mahlomaholo, my special aunty 'Mannana Sephoso and my grandmothers ‘Majanki Alice Mahlomaholo (Lt) and ‘Masechaba Mahlomaholo for their unrelenting love. They stimulated my creativity by listening to my expressed thoughts. Their undivided attention gave me respect for my own opinions, a respect that still exists today. Kea leboha Basia ba batle!

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List of acronyms

µ	Micrometre
a_w	Water activity
ATP	Adenosine triphosphate
CPGR	Centre of Proteomics and Genomic Research
EPS	Extracellular Polymeric Substance
ETC	Electron Transport Chain
FV	Fermenting Vessel
GC	Gas Chromatography
GRAS	Generally Regarded As Safe
HPLC	High Performance Liquid Chromatography
LAB	Lactic Acid Bacteria
MRS	de Man Rogosa and Sharpe
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (Reduced form)
OTU	Operational taxonomic unit
NUL	National University of Lesotho
PCA	Plate Count Agar
QS	Quorum Sensing
RBCA	Rose Bengal Chloramphenicol Agar
SEM	Scanning Electron Microscope
TEM	Transmission Electron Microscope
TLTC	Too Little To Count
VRBMA	Violet Red Bile with Mug Agar

Table of contents

List of tables

Chapter 2

Chapter 4

Chapter 5

List of figures

Chapter 2

Chapter 5

“Philosophy includes theory or investigation of the principles or laws that regulate the universe and underlie all knowledge and reality. Archaeology is the scientific study of the life and culture of ancient peoples. Anthropology is the study of races, physical and mental characteristics, distribution, customs, social relationships, and so on. When we start to study man’s foods, we become involved in all of these. In fact, when we study fermented foods, we study the most intimate relationships among man, microbes and foods”-Professor Keith Steinkraus

Chapter 1

About this study

1.1 Introduction

Fermentation is one of the oldest and economically efficient ways of preparing and preserving food. Records show that this method dates back millennia of years and substantial evidence has been seen on engravings of Egyptian tombs indicating that fermentation processes have a long history with mankind (Hammes *et al.*, 2005; Odunfa, 1988). Food fermentation is described as the invasion of food substrates by edible microorganisms whose metabolic activities modify food substrates through improving their texture, taste, aroma and digestibility .Cereal based fermentation is a common practise in Africa especially within a marginalised, low-income group where it is a preferred method of preservation (Anukam & Reid, 2009). In addition, cereal based fermentation has attracted scientific interest due to its great potential of enhancing food safety through inhibiting the growth and multiplication of pathogenic and spoilage microorganisms (Steinkraus, 2002). Furthermore, several health benefits such as reduction of cholesterol levels, risk of certain cancers, as well as improving the immune system and the microbial stability of the intestinal tract have been documented (Chilton *et al.*, 2015; Marco *et al.*, 2017; Rathore *et al.*, 2012; Todorov & Holzapfel, 2014). As substrates, cereal grains such as maize, sorghum, wheat and millet are used either singly or in combination to produce a fermented product.

Most indigenous cereal based fermentation processes are usually carried out spontaneously and without the addition of the commercial starter culture. Fermenting microorganisms come from the raw materials, utensils as well from a portion of a successful previous batch (back-slopping) (Achi, 2005). Traditional fermentation processes are artisanal in nature and developments have particularly been based on experience attained through trial and error by consecutive generations in households who have been using the technology to prepare foods for domestic or business purposes. Furthermore, this ancient technology is empirically carried out without a comprehensive knowledge of the underlying principles of the fermentation process. Such an approach poses a major pitfall in ensuring the safety of the fermented product (Benkerroum, 2013; Bigot *et al.*, 2015; Fnifst, 2008).

Lesotho is a country located in Southern Africa. The entire country is land-locked within South Africa (Majara, 2005). Lesotho's population is about 1.8 million, with 25% of the population distributed in the urban areas and 75% in the rural areas. People residing in rural areas still maintain the traditional lifestyle, mostly depending on vegetables and cereals for food. Due to lack of refrigeration facilities in these areas, fermentation of food substrates is thus the preferred method to preserve food (Gadaga *et al.*, 2013).

Among the variety of cereal based products in Lesotho, *sesotho* is a popular opaque traditional cereal based fermented beer and is usually consumed at cultural ceremonies as well as being prepared for village level business. With thus, the assumption is that it is not different from other cereal based fermented products found worldwide in terms of the organoleptic properties and health benefits as well as the pitfalls such as being produced under primitive conditions, poor quality, and short shelf-life associated with traditional cereal based fermented products.

The aim of this chapter is to explain the objectives and contribution of this study to the food fermentation fraternity as well as to give an outline of this thesis.

1.2 Objectives of this study and its contribution to the food fermentation fraternity

Traditional food fermentation is a vital indigenous knowledge that has been passed down from one generation to the next, especially in Africa. . Microorganisms are the main players in fermentation as they influence safety, nutritional value, health benefits and the flavour profile of the fermented product. This wealth of knowledge is however facing a challenge of being displaced by imported western foods with their glamorous image

The main objectives of this study are to document the indigenous knowledge on the fermented cereal based products in Lesotho. To investigate the microbial ecology, establish patterns followed, interactions and diversity during the fermentation process of *sesotho*. This study will also highlight the important and dominant groups of microorganisms in *sesotho* as well as their interaction during the fermentation process as well as the critical points for hazard analysis as the fermentation is progressing.

This study will contribute to the food fermentation fraternity as follows:

- Documentation will preserve this vital indigenous knowledge and enable the creation of a comprehensive database for future generations of microbiologists, biochemists, historians, nutritionists and food scientists.
- Documentation will also enable the technical developments of the processes which will encourage the feasibility of the industrialisation of the product with improved health benefits, nutrition, safety record and consistent flavour profiles.
- Characterisation of important dominant groups of microorganisms will enable the development of convenient starter cultures that harbour probiotic potential.
- Identification of uncultured and uncultivable important microorganisms involved during *sesotho* fermentation will contribute to an ever-expanding database of microorganisms involved in food fermentations world-wide.
- Understanding of the consensual microbial definition of *Sesotho* is a crucial initiative as it will contribute to opening new horizons about the microbial interplay during the cereal based fermentation processes in general.
- Identification of critical control points for hazard analysis during preparation and fermentation of *sesotho* will be of great importance as it will also enable the creation of database for policy makers as well as food regulatory officers within the government concerning the safety of the traditional fermented products.
- Examination of the microbial spatial arrangement during the fermentation process will give a clear picture of the microbial content and interaction during cereal based fermentations and this will give a crucial perspective regarding the development of the starter culture.

1.3 Thesis framework

The outline of this thesis is as follows:

Chapter 2 presents a brief overview of cereal based fermentation. The overview discusses the literature on: History of cereal based fermented food products and the nature of the process in section 2.2: Essentials for cereal based fermentations in section 2.3: Microbiology of cereal based fermented foods in Section 2.4: Biochemistry of cereal based fermented foods in section 2.5; African perspective on cereal based

fermented foods in section 2.6: Background of Lesotho and its fermented products in section 2.7: Benefits of cereal based fermented foods in section 2.8: Nutritional quality of cereal based fermented foods in section 2.9: Hygiene and safety risks associated with cereal based fermented foods in section 2.10: The future of cereal based fermented foods is discussed in section 2.11.

In chapter 3, the microbial patterns during the *sesotho* fermentation process are investigated and microrganisms quantified. Materials and methods are discussed in section 3.3: Results and implications are discussed in section 3.4. Chapter conclusion is discussed in section 3.5.

In chapter 4, is the metagenomics insight on the bacterial diversity and distribution during the *sesotho* fermentation. Materials and methods are discussed in section 4.3: Results and implications are discussed in sections 4.4-4.7. Chapter conclusion is discussed in section 4.8.

In chapter 5, is the Scanning Electron Microscopy examination of the earthen ware fermenting vessels used to prepare *sesotho*. Materials and methods are discussed in section 5.4. Results and implications are discussed in sections 5.5 and 5.6. Chapter conclusion is discussed in section 5.7.

Chapter 6 is general discussion and conclusions drawn from of the study

Chapter 7 is the overall summary of the study.

1.4 References

Achi, O. K. (2005). The potential for upgrading traditional fermented foods through biotechnology. *African J Biotechnol* **4**, 375–380.

Anukam, K. C. & Reid, G. (2009). African Traditional Fermented Foods and Probiotics. *J Med Food* **12**, 1177–1184.

Benkerroum, N. (2013). Traditional Fermented Foods of North African Countries : Technology and Food Safety Challenges With Regard to Microbiological Risks. *Compr Rev food Sci food Saf* **12**, 54–89.

Bigot, C., Meile, J.-C., Remize, F. & Strub, C. (2015). Applications of Metagenomics to Fermented Foods. In *Fermented Foods, Part I (Biochemistry Biotechnol*, pp. 333–346. Edited by C. R. Didier, Montet; Ramesh. CRC Press 2015.

Chilton, S. N., Burton, J. P., Reid, G. & Reid, G. (2015). Inclusion of fermented foods in food guides around the world. *Nutrients* **7**, 390–404.

Fnifst, O., 2008. *The Role of Traditional Food Processing Technologies In National Development: the West African Experience.* University of Ibadan.

Gadaga, T. H., Lehohla, M. & Ntuli, V. (2013). Traditional Fermented Foods of Lesotho. *J Microbiol Biotechnol Food Sci* **2**, 2387–2391.

Hammes, W. P., Brandt, M. J., Francis, K. L., Rosenheim, J., Seitter, M. F. H. & Vogelmann, S. A. (2005). Microbial ecology of cereal fermentations. *Trends Food Sci Technol* **16**, 4–11.

Majara, N. (2005). *Land Degradation in Lesotho : A Synoptic perspective*. University of Stellenbosch.

Marco, M. L., Heeney, D., Binda, S., Cifelli, C. J., Cotter, P. D., Foligné, B., Gänzle, M., Kort, R., Pasin, G. & other authors. **(2017).** Health benefits of fermented foods: microbiota and beyond. *Curr Opin Biotechnol* **44**, 94–102.

Odunfa, S. a. (1988). Review: African fermented foods: from art to science. *MIRCEN J* **4**, 259–273.

Rathore, S., Salmerón, I. & Pandiella, S. S. (2012). Production of potentially probiotic beverages using single and mixed cereal substrates fermented with lactic acid bacteria cultures. *Food Microbiol* **30**, 239–244.

Steinkraus, H. K. (2002). Fermentations in world food processing. *Compr Rev Food Sci Food Saf* **1**, 23–32.

Todorov, S. D. & Holzapfel, W. H. (2014). Traditional cereal fermented foods as sources of functional microorganisms. In *Adv Fermented Foods Beverages Improv Qual Technol Heal Benefits*, pp. 123–148. Edited by W. Holzapfel.

Chapter 2

Overview of cereal based fermented foods

2.1 Introduction

Food fermentation can be described as the biochemical modification of food substrates by edible microorganisms through their metabolic activities as well as their enzymes (Steinkraus, 2002). Food fermentation processes are intentionally carried out to transform food through improving acceptable sensory properties such as taste, aroma, texture as well as improving their shelf-life and the nutritive value (Achi & Ukwuru, 2015; Blandino *et al.*, 2003). Some of the most popular food products are derived from a wide variety of fermentable substrates such as cereal grains, fruits and vegetables as well as milk, fish and meat. Although these fermentable substrates may be seasonal, fermentation itself is climate independent and its by-products can be recycled as livestock feed (Dykes & Rooney, 2006; Fnifst, 2008; Marshall & Mejia-Lorio, 2012). In Africa, fermented food products play a role in cultural functions such as marriage and rain making ceremonies where they serve as intoxicating and thirst quenching drinks, they are also prepared for small-scale commercial purposes, funerals as well as weaning foods for children (Bassey *et al.*, 2013; MacDonald *et al.*, 2012; Motarjemi & Nout, 1996; Simango, 1997).

Fermentation activities can be integrated with other domestic activities and can particularly contribute to improving the livelihoods of women, landless poor and the disabled, who with proper access to training and access to inputs can increase their independence as well as income generation (Gadaga *et al.*, 2013; Marshall & Mejia-Lorio, 2012). This technique provides a cheap means to preserving and retaining the nutritional quality of food (providing much needed nutrients in diets) through destroying undesirable components such as toxins and as well as inhibiting a wide range of pathogenic microorganisms (Ahmad *et al.*, 2016). It is a common practice in rural areas and low-income households which lack food safekeeping facilities such as refrigeration (Motarjemi & Nout, 1996; Taiwo, 2009).

The traditional fermentation process is usually carried out mostly by women and it is performed without the addition of a defined starter culture. Fermentation is thus left to chance inoculation from the environment with fermenting microorganisms coming from

the utensils, raw materials or from the previous successful batch of a fermented product (this process is known as “back slopping”) (Holzapfel, 2002). Fermentation depends on the biological activity of fermentative microbes such as lactic acid bacteria (LAB), yeasts and moulds to produce metabolites that can impede the growth and survival of undesirable microflora in food products (Mufandaedza *et al.*, 2006; Yang *et al.*, 2012). These metabolic activities are not only important for food shelf-life and safety, but also play a role in developing characteristic properties such as aroma, texture, taste and appearance of the food product (Holzapfel, 2002; Kingamkono *et al.*, 1997; Motarjemi & Nout, 1996).

In this context, the objective of this chapter is to explore spontaneous cereal based fermentation as a house-hold technology for improved food and nutrition security. This chapter will also document the processing steps of fermented cereal based products in Lesotho.

2.2 History of indigenous fermented food products and nature of the fermentation process

The history of fermented foods and man comes a long way. This is the most intimate companionship between microorganisms and foods. Anthropologists have suggested that the main driving force behind food fermentation was prevention against spoilage, introduction of new varieties in diets as well the acceptable tastes and aromas that this process produces in foods (Motarjemi, 2002; Selhub *et al.*, 2014). There is substantial evidence that fermentation of food dates as early as the building of Great Wall of China in the 3rd century BC, from engravings on Egyptian tombs and they have long been part of tradition in African, Latin American, British and Chinese history (Anukam & Reid, 2009) .

Despite a long history between man and fermentation, the understanding of the science behind this art came quite late as the role of microorganisms was not yet appreciated or known at that time. It was only in 1857 when a French chemist and microbiologist Louis Pasteur showed that bacteria are involved in milk fermentation. This was the first step in clarifying the chemistry of fermentation. Pasteur described the process as “*la Vie sans”*, or “life without air” (Bourdichon *et al.*, 2012). Fermentation carried out without the presence of oxygen is an anaerobic process and the microorganisms that thrive under these kinds of conditions are termed “obligate

anaerobes" and those that can thrive with or without the presence of air are termed "facultative aerobes" (Khalid, 2011). It was only in 1896 when the roles of enzymes in fermentation were understood following the experiments that were carried out by German chemists Hans and Eduard Bucher. In 1907 the Russian microbiologist Ellie Metchnikoff isolated *Lactobacillus* from fermented milk (Odunfa, 1988).

Most energy-conserving reactions in living microorganisms are based on the transfer or exchange of electrons between substrates. Such reactions entail reduction (gain of electrons) and oxidation (loss of electrons) and are collectively known as "redox" reactions. During these reactions, one substrate is reduced and concomitantly the other is oxidised and vice-versa, sometimes the same substrate is both reduced and oxidised. Living microorganisms carry out redox reactions differently in a sense that others are aerobic, others anaerobic and others are able to thrive under both conditions. Microorganisms can also be grouped according to the manner in which they carry out this redox reactions in a sense that others respire whereas others ferment, as well as on the type of substrate which they utilise, whether organic or in organic (Caplice & Fitzgerald, 1999; Muller, 2003).

In respiring aerobes, oxygen is the terminal electron acceptor whereas in respiring anaerobes, the electron acceptor can be both organic and inorganic. In respiring microorganisms both aerobic and anaerobic, energy in the form of Adenosine Triphosphate (ATP) is produced by an electron transport chain (ETC). However, this is not the case in fermentation as most of the ATP is produced by substrate level phosphorylation. Although fermentation is described as anaerobic redox process, it encompasses both anaerobic and aerobic processes, in which an oxidation of one substrate is coupled to the reduction of another, with the difference in the redox potential between the substrate and the end-product providing energy for ATP production (Muller, 2003). However, most of the fermentations use the same substrate both as an oxidant and reductant.

Preservation of foods by fermentation depends on the principle of carbohydrate oxidation and its relativeness to generate the final products which generally are organic acids, carbon dioxide and alcohol. These metabolic end products inhibit both pathogenic and spoilage microorganisms and since oxidation is only partial, the food retains some energy potential to provide much needed nutritional benefits to the

consumer (Kandler, 1983; Khalid, 2011). The nature of the fermentation end products varies with species and their respective pathways are named depending on the end products obtained. For instance, alcoholic, lactic, acetic and alkali fermentations (Steinkraus, 2002). The Biochemistry of lactic acid fermentation will be discussed later in this overview.

Due to absence of a writing culture in most parts of Africa during those times (ancient time), the origin of the fermented foods in Africa is difficult to trace. During the process of time, Muslim and Arabs conquered most of western and northern parts of Africa that was when many records and documentations on fermented foods were made. These records were made by Arab travellers who were mostly geographers and merchants. By that time (from the 8^{th} to the16^{th} century), the art of fermentation had been perfected as well as being incorporated as part of people's culture (Odunfa, 1988). Regrettably, this knowledge (recording/documenting) brought by the Arabs was not passed on to the forested west and central Africa even as far extending to the southern parts of Africa.

2.3 Essentials of cereal fermentations

Stored cereal grains are metabolically inactive in their raw natural state, this is mainly due to the low water activity (a_w) which is equal or below 0.6. Because of this low water activity, nutrients in cereals are not readily available for microorganisms and the enzymes. Before fermenting cereal grains, they have to be milled, this action aids in increasing the surface area to allow for the microbial and enzymatic (amylases, lipases and proteinases) action. To ferment, milling will then be followed by the addition of water, this increases a_w and exposes the nutrients for microbial action and also activating the endogenous enzymes (Hammes *et al.*, 2005). It is the addition of water that significantly influences the ecological factors within the cereal matrix. Upon an increase in a_w, a redox potential occurs by respiration, there will be a decrease in pH by respiration and by fermentation (Achi & Ukwuru, 2015; Blandino *et al.*, 2003). The substrates will then become available for endogenous enzymes to act on, their availability is owed from the physiological activities of microorganisms within the cereal grains or those that have been intentionally added (starter culture or back-slopping). These events will cause a spontaneous change of the ecological state in the cereal

matrix. The spontaneous biochemical changes during the fermentation process will then lead to the final product with desired organoleptic characteristics.

According to Achi & Ukwuru (2015), fermentation processes and the quality of an end-product are dependent on a number of variables such as:

- The type and quality of cereal being fermented. Cereal grains vary in nutrient, growth factor and mineral content, as well as the efficacy of growth inhibiting principles.
- The degree of comminution of the grains. This encompasses the surface area available for the microorganisms to act on in order to produce some substrates that endogenous enzymes can convert to characteristic compounds such as alcohol, lactate, organic acids, etc.
- Water content. Different microorganisms function at different a_w, so the availability of water will determine which microorganisms start to grow and multiply on the substrate.
- The fermenting temperature. The functionality of microorganisms within food matrices is also reliant on the cardinal temperatures. Different microorganisms require different temperatures to function, so temperature will determine which microorganisms participate during the fermentation process.
- The duration of the fermentation process. During fermentation, there is a decrease in pH, the pH can drop as low 3.2 and as high as 4.5. So the fermentation duration is very critical because potential pathogenic, spoiling as well as some beneficial microorganisms cannot survive acidic environments. The longer the fermentation the more decrease in pH and increase in alcohol content of the fermented product as well rise in other chemicals that can cause off-flavours. As such the fermentation period should be monitored carefully in order to get the desired end-product and inhibit potential pathogenic and spoilage microorganisms without having to compromise the nutrition, desired sensory characters as well as the probiotic potential of the end-product.

2.4 The microbiology of lactic fermented foods

The art of fermenting foods has been practiced for thousands of years with the main drive being to preserve as well as to introduce new flavours, textures and aromas on fermented food products (Ali & Mustafa, 2009; Franz *et al.*, 2014; Tamang *et al.*, 2016; Zorba *et al.*, 2003). Of course this process is artisanal in nature and the role microorganisms played in fermentation may have not been understood and appreciated. Fermentation was practised at house-hold level at that time and was mainly carried out by women. The development of this technology was by trial and error as there was no sound knowledge of the science behind this technology. During the process of time, records and documentations of these practices were made, giving birth to the science behind the fermentative processes (Blandino *et al.*, 2003). During this evolution, it was revealed that fermented foods carry a better nutritional value and contain far less potential spoilers and pathogens as compared to their unfermented counterparts (Mokoena *et al.*, 2016). So this prompted even more curiosity on the role of microorganisms behind fermentation as a household-level food-processing technology (Holzapfel, 2002; Schillinger *et al.*, 1996; Steinkraus, 1983).

Most food fermentations are a result of microbial interactions, either working together simultaneously or in sequence. For example, *umqombothi* is produced from a partnership between LAB bacteria and yeasts. The LAB dominates the initial stages and then yeasts take over the final stages. The LAB through decreasing the pH and turning conditions to become anaerobic creates an ideal environment for yeasts to perform their function in producing alcohol (Katongole, 2008). Extensive studies have shown that a microorganism that starts the fermentation will grow up until its growth and metabolic activity are inhibited by the by-products. During the period of initial growth, there is a development of other microorganisms that are going to take over when the already created conditions begin to inhibit the former microorganisms (Mufandaedza *et al.*, 2006; Narvhus & Henry, 2003). Generally, smaller microorganisms are the ones that multiply quickly and thus taking up the nutrients from the surrounding rapidly. Since bacteria are the smallest of microorganisms, they will grow faster, followed by yeasts and moulds (Giraffa, 2004).

Fermented foods have been defined as those foods that have been invaded by edible microorganisms that possess enzymes such as amylases, lipases and proteases that

break down polysaccharides, fats and proteins to non-toxic products with aromas, textures and flavours that are pleasant and attractive to the human consumer (Steinkraus, 2002).. Biochemical reactions that take place during fermentation improve the nutritional value of a food product through improved digestibility and increased vitamin levels (Lin & Gänzle, 2014; Rakin *et al.*, 2007; Rhee *et al.*, 2011; Todorov & Holzapfel, 2014). Toxins and anti-nutritional compounds that may be contained in many raw materials (cereals, legumes, fruits and vegetables) can be eliminated by the biochemical activities of microorganisms during fermentation (Caplice & Fitzgerald, 1999). The microbial action may also lead to the destruction of undesirable compounds such as polyphenols, tannins and phytates (Dykes & Rooney, 2006). The safety of the fermentation process is dependent on the microbial activity to produce the metabolites that can suppress or inhibit the growth of undesirable micro-flora in foods. Microorganisms present in fermented foods are mixtures of cultures of yeasts, fungi and bacteria. These microbial communities may interact in parallel or in a sequential manner with exchanges in dominant microbiota during fermentation (Giraffa, 2004). Research has revealed that yeasts are also present in several different indigenous fermented foods and beverages. However, their role in the food products has not been extensively investigated. LAB is the predominant bacteria in fermented foods; they hold the Generally Regarded as Safe (GRAS) status and promise for selection against undesirable microorganisms and implementation as protective cultures (Holzapfel, 2002; Schillinger *et al.*, 1996).

To acknowledge the role played by microorganisms in fermentation, it is imperative to understand the key elements such as which microorganisms are present, what are their by-products, what is the impact of those by-products as well as understanding the microbial succession during the fermentation process (Caplice & Fitzgerald, 1999; Steinkraus, 2002). Extensive research on food fermentations unveiled that in order to get a consistent end-product; it is desirable to be able to control the succession of microorganisms or specific microorganisms that dominate the food microbiota, which is basically the basis of the starter culture development (Ali & Mustafa, 2009; Amenan *et al.,* 2014; Holzapfel, 2002; Smid & Lacroix, 2013).

2.4.1 Starter culture concept

Indigenous fermented products undergo spontaneous fermentation and are artisanal in nature, produced as a result of back-slopping where a small portion of previously successful batch is utilised to initiate a new fermentation. In this process, microbial cultures that are present are passed between generations and from household to household (Marsh *et al.*, 2014). Although fermentation is driven by microbial activities, knowledge on the exact microbial content in indigenous spontaneous fermented products is tricky as it depends on several factors such as the microbial load on the raw materials, hygiene of producers, type and treatment of the fermentation vessel as well as the length of fermentation (Holzapfel, 2002; Marsh *et al.*, 2014).

In broad terms, a starter culture may be defined as a preparation containing high numbers of live microorganisms, which may be added to bring desirable modifications in a food substrate (Caplice & Fitzgerald, 1999). Adaptations of such starters to the food substrate will serve to speed up the fermentation process, enable a stricter control of the fermentation process and thus giving a more predictable outcome (Vuyst, 2004). Starter cultures are specifically selected for a substrate or raw material and the metabolic abilities of the selected strains are used to support the technical process as well as obtaining a desired quality of the end-product. Adaptation of the starter to the substrate as well as other aspects such as texture and flavour improvements also serve as the criteria for selection and as an improvement to a rather basic and empirical approach of back-slopping (Holzapfel, 1997, 2002; Nout, 2009). Most common groups of microorganisms contained in the starter cultures comprise of bacteria, yeasts and moulds.

2.4.2 Moulds

Moulds are important organisms in food, both as preservers and spoilers. Species of *Aspergillus, Actinomucor, Mucor, Monascus, Neurospora, Parcillomyces, Penicillium, Amylomyces* and *Ustilago* have been reported from many fermented foods (Iacumin *et al.*, 2009).There are some moulds that produce toxins and those that play a contributory role to the spoilage of foods. Often, *Aspergillus* species are responsible for undesirable modifications in foods. These moulds are commonly found in foods with high concentrations of sugar and salt as they can tolerate such environments. Nonetheless, certain moulds are responsible for characteristic flavours to foods and

others produce enzymes that degrade anti-nutritive factors (Tamang *et al.*, 2016). *Penicillium* species are associated with ripening as well as imparting characteristic flavours to cheeses. Moulds are aerobic organisms and are therefore require oxygen in order to grow. This group of microorganisms possess the greatest array of enzymes and thus can proliferate on most types of foods (Iacumin *et al.*, 2009). Despite being able to colonise most food types, moulds do not play a significant role in the desirable changes of fermented vegetable, cereal and fruit products in Africa (Holzapfel, 1997).

2.4.3 Yeasts

Yeasts are defined as unicellular ontogenic stadia of true fungi, they reproduce sexually and asexually. The asexual reproduction is by means of budding and fission. Yeasts are distributed widely in nature; present in vineyards, orchards, in the air, in the soil as well as in the intestinal tract of animals. Just like moulds and bacteria, yeast can also have beneficial and harmful effects on foods. In fermentations yeasts are treasured for their ability to produce alcohol from simple sugars such as glucose, as well as producing some acceptable organoleptic properties to the consumers (Hammes *et al.*, 2005; Mugula *et al.*, 2003; Nout *et al.*, 1995).

Genera of yeasts reported to be common in fermented cereal based food products are *Candida*, *Brettanomyces, Geotrichium, Cryptococcus, Hansenula, Debaromyces, Issatchenkia, Pichia, Saccharomyces* and *Rhodotorula* (Tamang *et al.*, 2016; Watanabe *et al.*, 2008). Among these different genera, *Saccharomyces* and *Candida* are commonly detected in spontaneous alcoholic fermentations i.e. African traditional beers (Benkerroum, 2013; Greppi *et al.*, 2013). *Saccharomyces cerevisiae* has been extensively exploited in the industrial production of western-style beers and wines. Dehydrated yeast is also commercially available throughout Africa, for bread making; however, it is also employed for traditional opaque beer brewing.

2.4.4 Bacteria

The widelyrepresented groups of LAB reported in fermented beverages and foods are those from the genera of *Enterococcus, Lactobacillus, Leuconostoc, Lactobacillus, Streptococcus, Lactococcus, Oenococcus, Pediococcus, Carnobacterium* and *Wiesella* (Tamang *et al.*, 2016). LAB isolated from fermented food products produce organic acids and a wide range of antimicrobial agents such as bacteriocins and enzymes of importance. This group of bacteria is thus treasured for enhancing shelf-

life, microbial safety and texture to the final product. They also contribute to pleasant aromas and flavours. LAB are treasured for their reduction of toxic or anti-nutritive factors (Mokoena *et al.*, 2016; Vuyst, 2004).

LAB have been reported as the most dominant group of microorganisms in cereal based fermented food products. Thus signalling a beneficial intimate companionship between LAB, food and the human environment. Studies have shown that these beneficial interactions; both in food and in intestinal tracts of humans, combined with the long tradition of lactic fermented foods in many cultures have strengthened the general conclusion that this group of bacteria may be Generally Regarded as Safe (GRAS) (Achi & Ukwuru, 2015; Mokoena *et al.*, 2016).

2.4.5 Mixed cultures

Spontaneous fermentations result from the metabolism of a variety of microorganisms. During spontaneous fermentations, there are competitive activities exhibited by different groups of microorganisms (yeasts and bacteria). As a result, strains that adapt best to the fermentative conditions will thus be predominant during the particular stages of the process (Achi & Ukwuru, 2015; Assohoun-Djeni *et al.*, 2016). This exchange or switch in dominance is due to several factors such as competition for growth nutrients or may produce metabolic products that inhibit each other's growth. For example, several studies on the microbial interactions during cereal based fermentation have indicated that LAB usually dominate the early stages of the process and yeast usually take over the final stages (Katongole, 2008; Muyanja *et al.*, 2003). Other studies also indicated that yeasts are present in several traditional lactic fermented foods and they often contribute to the desirable sensory properties by virtue of their metabolic activities as well producing the required alcohol in alcoholic beverages (Marsh *et al.*, 2014).

2.5 The biochemistry of lactic acid bacteria in cereal based lactic fermented foods

Extensive research on cereal based fermentations have revealed that LAB play a crucial role in the final product formation through enhancing taste, texture, shelf-life, safety, nutrition and some organoleptic properties acceptable to the consumer (Deegan *et al.*, 2006; Marco *et al.*, 2017; Oyedeji *et al.*, 2013; Todorov & Holzapfel, 2014). LAB is made up of a diverse group of non-spore forming, non-motile rods and

cocci shaped, gram-positive, catalase negative organisms. This group of bacteria are generally mesophilic yet able to proliferate at temperatures as low as 5°C and even as high as 45°C. While the majority of these strains are able to grow at pH levels of 4 to 4.5, some strains are able to grow at low pH levels of 3.2 and others even as high as pH of 9.6. LAB strains are chemoorganotrophic and only proliferate in complex media; organic compounds (hexoses) are employed as energy sources to produce lactic acid (Caplice & Fitzgerald, 1999). Since LAB strains lack a functional heme that is linked to the ETC, they use substrate level phosphorylation to synthesize energy (In the form of ATP). LAB strains have the ability to degrade hexoses (six carbon sugars) to lactic acid as the sole product (homofermentative) or lactic acid with additional products such as ethanol, formic acid, acetic acid, carbon dioxide or succinic acid (heterofermentative) (fig. 2.1) (Muller, 2003). This difference is due to different metabolic pathways that are employed for glucose oxidation. Lactic acid produced may be of two stereoisomers; i.e. **L** (-) or less frequently, **D** (-) or can be a mixture of both. Documentations have been made revealing that humans cannot metabolise the **D** (-) lactic acid and it is not recommended for children and infants (Motarjemi & Nout, 1996)

2.5.1 Homofermentative pathway

Homofermentative bacteria are able to transform almost all sugars that serve as substrates especially the conversion of glucose into lactic acid as a sole product. Homofermentative pathway includes glycolysis that leads from hexoses to pyruvate. In this metabolism pyruvate serves as an electron acceptor and is reduced to lactic acid by the action of the enzyme lactate dehydrogenase. This enzyme will then catalyse a stereospecific reduction to either **L** (-) or **D** (-) lactate (Muller, 2003) (fig. 2.1).

2.5.2 Heterofermentative pathway

Compared to homofermentative bacteria, heterofermentative bacteria lack the aldolase enzyme but instead contain phosphoketolase. In this pathway glucose-6-phosphate (G-6-P) is oxidized to 6-phosphogluconate then decarboxylated to ribulose 5-phosphate. Ribulose 5-phosphate can be epimirized to either xylulose-5-phosphate or to ribose-5-phosphate. Following epimirization, xylulose-5-phosphate is then cleaved to form glyceraldehyde-3-phosphate (G-3-P) and acetyl phosphate.

The G-3-P is then metabolised into lactic acid through following the same rout as in the homofermentative pathway. The other cleaved portion which is acetyl phosphate, has two possible destinations, which depend on the environmental conditions. One possible destination is the production of acetic acid through acetate kinase enzyme. The end products of this pathway are lactic acid and acetic acid. The other possible destination is that acetyl phosphate can be successively reduced into ethanol, in which then the molecules of coenzyme NADPH formed during the two oxidation reactions of glucose from the beginning of the heterofermentative pathway are reoxidised. This re-oxidation reaction is of paramount importance for regenerating the necessary coenzymes in this pathway. The end products are lactate, carbon dioxide and ethanol (Muller, 2003).

Figure 2.1: Flow diagram illustrating the homofermentative and heterofermentative metabolic pathways (Muller, 2003).

2.6 African perspective on cereal fermentations: A wide diversity

Cereals are more widely consumed as major food source in African countries than in developed parts of the world (Anukam & Reid, 2009). Most cereal based foods throughout Africa are processed by spontaneous or natural fermentation. Fermented cereals are particularly important as they are used as dietary staples by adults and as weaning foods for infants (Odunfa, 1988).

Alongside salting and drying, cereal fermentations date back thousands of years as one of the oldest forms to preserve food. Indicating that cereal fermentation has long been embedded in traditional cultures and village life in developing countries (Odunfa, 1988). Since cereals are seasonal, it is believed that fermentation processes were developed over the years by women of specific communities, with the objective to preserve food for times of scarcity, to impart desirable sensory properties to food products or to reduce toxicity (Rolle & Satin, 2002).

There is a wide diversity of cereal based fermented foods throughout Africa that have been documented, they can be classified based on their texture or their raw cereal ingredients (Table 2.1). This wide diversity of fermented foods varies according to factors such as geographical area, availability of raw material and cultural patterns

Table 2.1: An illustration of cereal based products classification (Odunfa, 1988).

BY RAW INGREDIENT	**EXAMPLE**
Sorghum based	Bushera, Burukutu
Millet based	Ogi, Pito
Wheat based	Bogobe
Maize based	Mahewu, Tsoeu-koto
BY TEXTURE	**EXAMPLE**
Liquid (Gruel)	Mahewu, Ogi
Solid (Dough)	Kenkey, Agidi
Dry (Bread)	Bogobe, Injera

2.6.1 Fermented cereal based food products in Africa

There is a broad diversity of indigenous fermented cereal food products such as porridges, beverages (alcoholic and non-alcoholic) and breads (Table 2.2). In Africa, some of these foods are consumed as major staples, breakfast, inebriating beverages, intoxicating beverages as well as weaning foods for children (Gadaga *et al.*, 2013; Katongole, 2008; Kayodé *et al.*, 2011; Nout, 2009; Rolle & Satin, 2002) .

Table 2.2: Various cereal based fermented food in Africa.

FERMENTED PRODUCT	RAW MATERIAL	REGION	MICROORGANISMS IMPLICATED	REFERENCES
Ogi	Maize/sorghum /millet	West Africa	*Lactobacillus* spp., *Saccharomyces* spp., *Candida* spp	(Kuye & Sanni, 1999)
Gari	Cassava	West Africa	*Lactobacillus* spp., *Saccharomyces* spp., *Candida* spp	(Ijabadeniyi, 2007)
Bushera	Sorghum/millet	East Africa	*Lactobacillus* spp., *Stretococcus* spp., *Leuconostoc* spp., *Pediococcus* *Saccharomyces* spp.,	(Muyanja *et al.*, 2003)
Burukutu	Sorghum/millet	West Africa	*Acetobacter* spp., *lactobacillus* spp., *Candida* spp.,	(Kayodé *et al.*, 2011)
Pito	Sorghum/millet	West Africa	*Acetobacter* spp., *Lactobacillus* spp., *Saccharomyces* spp., *Candida* spp.,	(Achi & Ukwuru, 2015)
Agidi	Maize/sorghum /millet	West Africa	*Pediococccus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *streptococcus* spp., *Saccharomyces* spp., *Candida spp.,*	(Amenan Anastasie Soro-Yao, Kouakou Brou, Georges Amani, 2014)
Umqombothi	Maize/sorghum	Southern Africa	*Lactobacillus* spp., *Saccharomyces* spp., *Candida* spp.	(Katongole, 2008)
Chibuku	Sorghum	Southern Africa	*Lactobacillus* spp., *Saccharomyces* spp.	(Mokoena *et al.*, 2016)
Togwa	Cassava/maize /sorghum/millet	East Africa	*Lactobacillus* spp.,*Leuconostoc* spp., *Pediococcus* spp., *Candida* spp., *Saccharomyces* spp.	(Mugula *et al.*, 2003)

2.7 Background of Lesotho and its fermented cereal based products

As previously mentioned in chapter 1, Lesotho is a country located in Southern Africa and completely land-locked within South Africa. Its terrain is predominantly mountains, which run mainly from south-west to north-east (fig. 2.2). The mountains take about 75% of Lesotho's area. The entire country lies above 1,000 metres (3,281 ft.) above sea level in elevation. Its lowest point is at 1,400 metres (4,593 ft.) above sea level, the highest lowest point of any country. Its climate zone thus can be classified as continental (Gadaga *et al.*, 2013; Majara, 2005). By virtue of this elevation, Lesotho's climate is thus cooler than most other regions of the same latitude. Lesotho's population is about 1.8 million, with 25% of the population distribution in the urban areas and 75% in the rural areas (Gadaga *et al.*, 2013). People in the rural areas still maintain the traditional lifestyle, depending on vegetables and cereals for food. Due to lack of refrigeration facilities in these areas, fermentation of food substrates is used as the preferred method of food preservation (Gadaga *et al*., 2013).

Although fermentation is an ancient method to preserve food, this indigenous technology is still part of the cultural norm and is usually practised at local village-level house-holds (Gadaga *et al.*, 2013). Indigenous fermented foods and beverages have generally been important in Lesotho as they constitute one of the main dietary components. Like that of many traditional fermented products, there is a decline in their appreciation as they are classified as poor man's food for their unstandardized unhygienic preparation techniques as well as perceived short shelf-life. Due to this lack of appeal, many people especially in urban areas have gravitated towards the imported and exotic food products. This is mainly because of their attractive appearance, long shelf-life, provided nutritional profile, as well as the social class associated with them. However, despite the stigma associated with fermented foods, extensive research has shown that fermentation has some benefits such as enhancing shelf-life, nutrition, and probiotic potential (Mokoena *et al*., 2016).

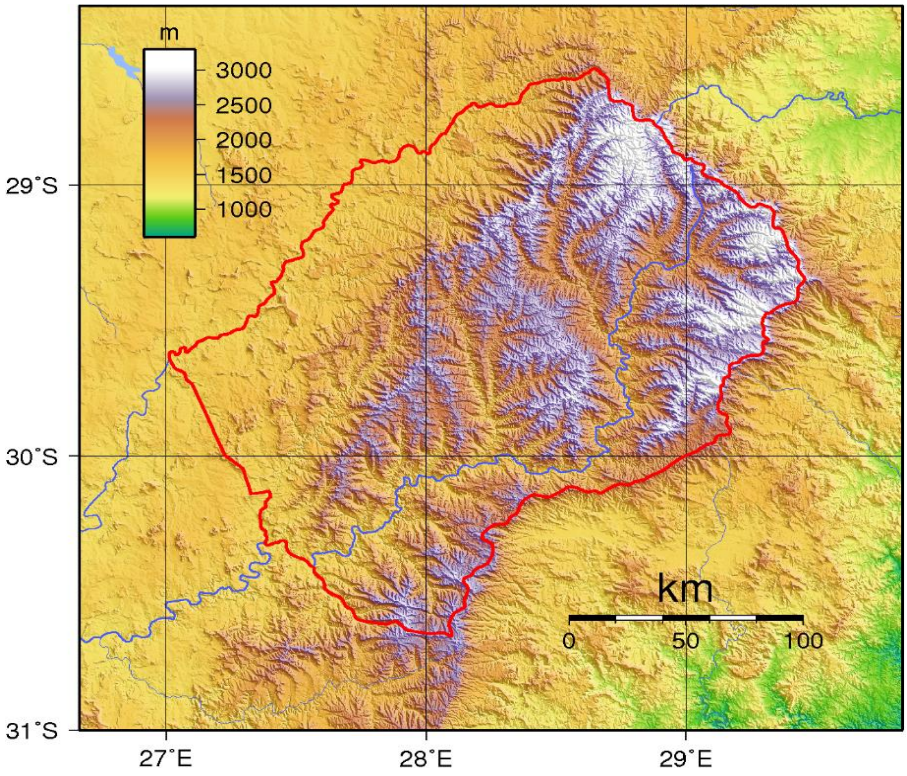

Figure 2.2: A topographical map of Lesotho.

2.7.1 Diversity of fermented cereal based products in Lesotho

In Lesotho, like in most African countries, preparation of indigenous fermented foods and beverages is mainly practised as a household art as they are produced in homes, and for village-level business. Consumption patterns and production rates of different cereal based fermented foods vary depending on the availability of raw materials and purpose of production. Unlike fermented alcoholic beverages that are mostly prepared for feasts and for village-level business whereby the particular cereal required can be sourced from elsewhere, the preparation of staples and weaning foods at house hold level on a daily basis depends on availability of the required cereal.

Currently, there is no adequate documentation on Lesotho's indigenous cereal based fermented products as well as lack of information regarding their microbial ecology (Gadaga *et al.*, 2013). If the fermentation conditions of these products are to be optimised and ultimately produced at an industrial level, the processes should be systematically studied and documented, the ingredients should be quantified and preparation conditions required for a successful fermentation identified. The availability of this vital indigenous knowledge will form part of a comprehensive database within the ethnic food fermentation fraternity as well as for historians

(Chelule *et al.*, 2010). With this scope as the background, the objective of this section is to do an in depth documentation of the cereal based fermented products in Lesotho.

Fermented food products produced in Lesotho have been documented (Gadaga *et al.*, 2013). However, the authors' findings were scanty as their survey was limited to Roma valley in the district of Maseru. Therefore in an effort to put together a comprehensive documentation on this vital indigenous knowledge of cereal based products across the country, information was sourced from local brewers, historians, local chiefs and senior citizens from five districts of Lesotho, namely Mafeteng, Butha-Buthe, Mokhotlong, Maseru and Thaba-Tseka representing the southern, northern, eastern, western and the central regions of the country respectively (fig. 2.2). This initiative was mainly driven by different recipes (usually conserved from region to region). Households producing alcoholic beverages for sale could be identified by a flag situated by the concerned household (fig. 2.3)

Figure 2.3: Households producing beer for village level commercial purposes identified by a flag.

2.7.2.1 Sesotho

Sesotho is a popular opaque traditional beer prepared in Lesotho. This alcoholic beverage is turbid and has a thin (becomes thin after being sieved) consistency. It is prepared as an inebriating drink at funerals, marriages, thanks giving gatherings and other cultural ceremonies. Nonetheless, *sesotho* is mostly prepared at village-level for commercial purposes.

Sesotho is produced from pure maize, sorghum or wheat flour, or sometimes a mixture of these flours (depending on the availability of cereal grains or the producer's discretion). The preparation of *sesotho* involves hand mixing maize/sorghum flour with wheat flour (fig. 2.4). A bit of warm water is mixed in to form a thick paste, followed by addition of boiling water to form a thinner paste. The paste is cooled and *tomoso* (liquid starter obtained from the previous successful batch of the initial fermentation) is added, the amount of starter added varies from household to household as it depends on its strength (tested by its sourness) and the amount of beer being produced. The vessel is covered and left overnight to ferment (fermentation length depends on the temperature (which takes longer during winter) and strength of the starter). Once it has fermented overnight it is called *lekoele*. *Lekoele* is then cooked, first the liquid upper phase is boiled. The remaining liquid and solid phases are then mixed by slowly stirring and poured over the boiling liquid. *Lekoele* is generally allowed to boil for 2-3 hours.

Following boiling, the mixture attains a thick consistency and is called *setoto*. *Setoto* is left to cool and when cool, *mmela* (sorghum malt) and *tomoso* also known as *kokola* or *moroko*, (fig. 2.5A) (spent solid starter obtained from the previous successful batch) are added. The fermentation vessel is then covered and left overnight in order to allow fermentation to proceed. Figure 2.5B depicts a sample of actively fermenting *sesotho* beer. Following fermentation, the mixture is sieved thoroughly prior to consumption. Due to different beliefs, in urban areas (western regions) brewers usually add sugar to the finished product, with a belief to produce a more potent beer. In the central regions, brewers produce *sesotho* beer mainly, if not only from wheat, and this is because wheat is the most abundant cereal grain in this region. Still on the lineof different beliefs, in contrast to urban citizens who just close the vessel to allow fermentation following the necessary preparation procedures, rural citizens burn a

piece of paper over the brew, while burning it is moved over the brew in a circular motion until it dies out. The paper ashes are dropped into the brew, the process is called *“Ho cheseletsa”.* The brewers explained that this technique is meant to encourage a smooth/non-flop fermentation process.

People staying in the eastern and western regions of Lesotho have an alternative shorter method of brewing s*esotho*, which involves a single fermentation step, in this method a mixture of wheat flour and sorghum meal is mixed with warm water to form a thin paste, then the paste is cooked (boiled for about 2-3 hours). The cooked mixture is called *lesheleshele*, (the name commonly given to unfermented porridges prepared from maize or sorghum meal). Following cooking the porridge is left to cool then *mmela* and *moroko* are added prior fermentation. The mixture is then allowed to ferment overnight. The following day it is sieved and the beer is ready for consumption. Whenever *setoto* and *moroko* are available, they can be consumed at house hold level. *Setoto* is consumed as porridge or may be mixed with maize meal pap (soft porridge) to give it a smooth texture. *Moroko* it is steamed before is consumed. On the other hand, *moroko* can also be used to feed the livestock.

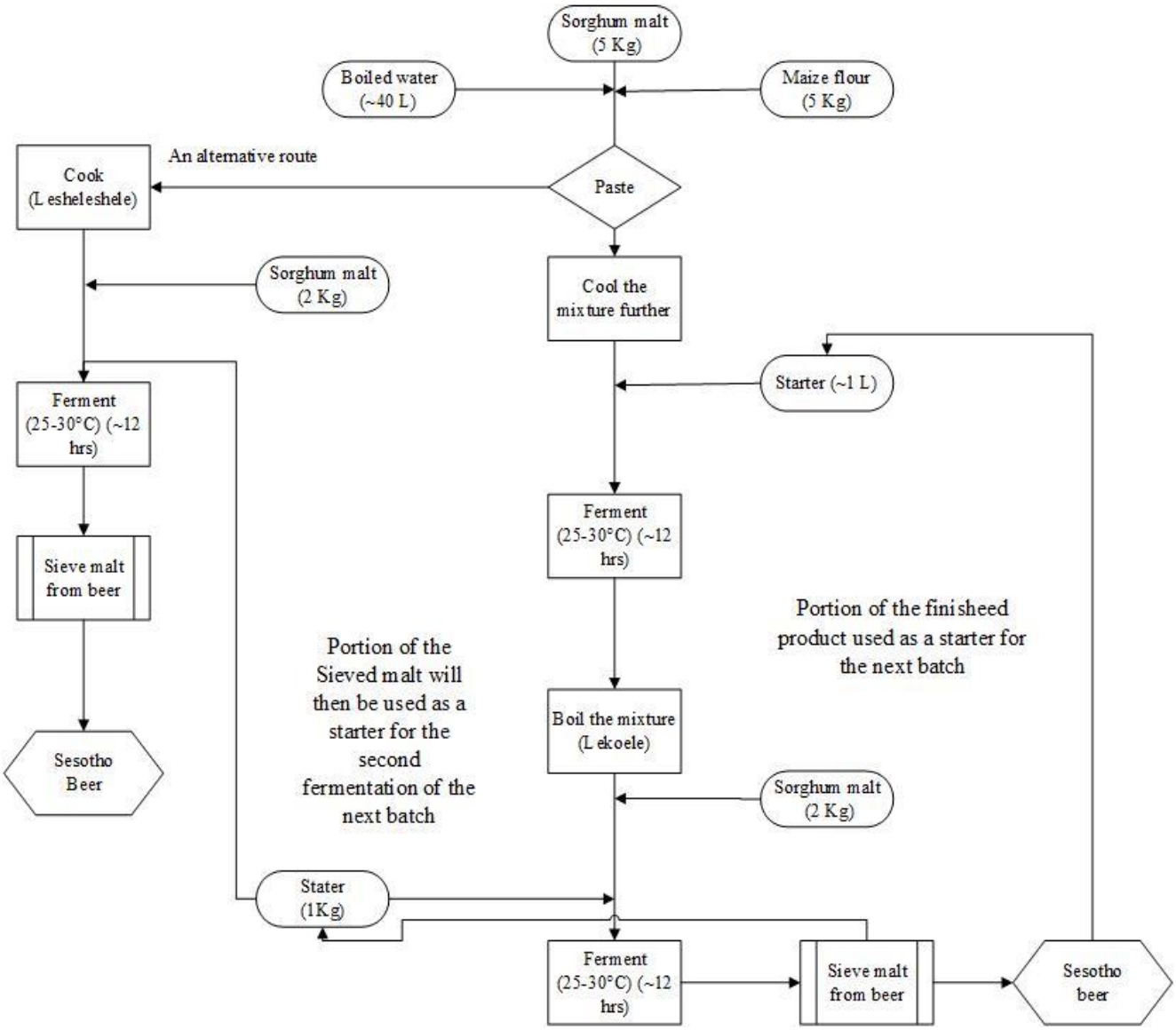

Figure 2.4: A flow diagram illustrating *sesotho* preparation (~30 L). ***The amounts of ingredients have been estimated.**

Figure 2.5: Spent solid starter (*Moroko*) obtained from the previous successful batch of final fermentation (A) and an actively fermenting *sesotho* beer (B).

2.7.2.2 Motoho

Motoho is the most widely consumed non-alcoholic fermented gruel in Lesotho, it is produced from sorghum flour. The series of events followed in the preparation of *motoho* are as shown in Figure 2.6. This popular gruel is consumed at household level as a thirst quencher, but may also be added to and mixed with maize meal pap to give it a smooth texture. *Motoho* is used as a special treat for visitors who may be passing by, or visitors coming to give condolences whenever there is a funeral or visiting the family to ask for a hand in marriage. It is also used as a weaning food for infants.

The preparation of *motoho* involves mixing flour with water (ambient temperature) to form a paste, warm water is then added to the paste to give it a thinner consistency. *Tomoso* (traditional starter culture obtained from back-slopping) or sometimes *lekoele* or *mohlaba* (traditional leftover beer) is then added to the mixture. Whenever *tomoso* is not readily available at house-hold, a stainless steel spoon or any other small kitchen utensil (stainless steel) is added to the thin paste which is then left for about 3-4 hours or overnight in winter (usually covered in a blanket) to ferment. After fermentation, *motoho* is cooked prior to consumption. Cooking involves boiling part of the upper liquid component of the brew, followed by mixing the remaining liquid with the paste at the bottom of the fermentation vessel and pouring over then allowing to cook for 2-3 hours, *motoho* has a lesser sour sensory property than *sesotho* and other alcoholic

products, which is explained by the little fermentation time it undergoes. To prepare *motoho* of a finer texture an alternative route as shown in Figure 2.4 is followed. This method is usually carried out in the rural areas. When those residing in the urban areas were asked if they know that alternative route of preparing a finer product, they indicated that they know the method but are however not practising the route as it is more labour intensive. This beverage is not filtered after fermentation, thus explaining its semi-thick gruel characteristic. Due to its popularity and a strong cultural attachment amongst people of Lesotho, *motoho* has found its place in the local food markets especially in the urban areas (fig. 2.7).

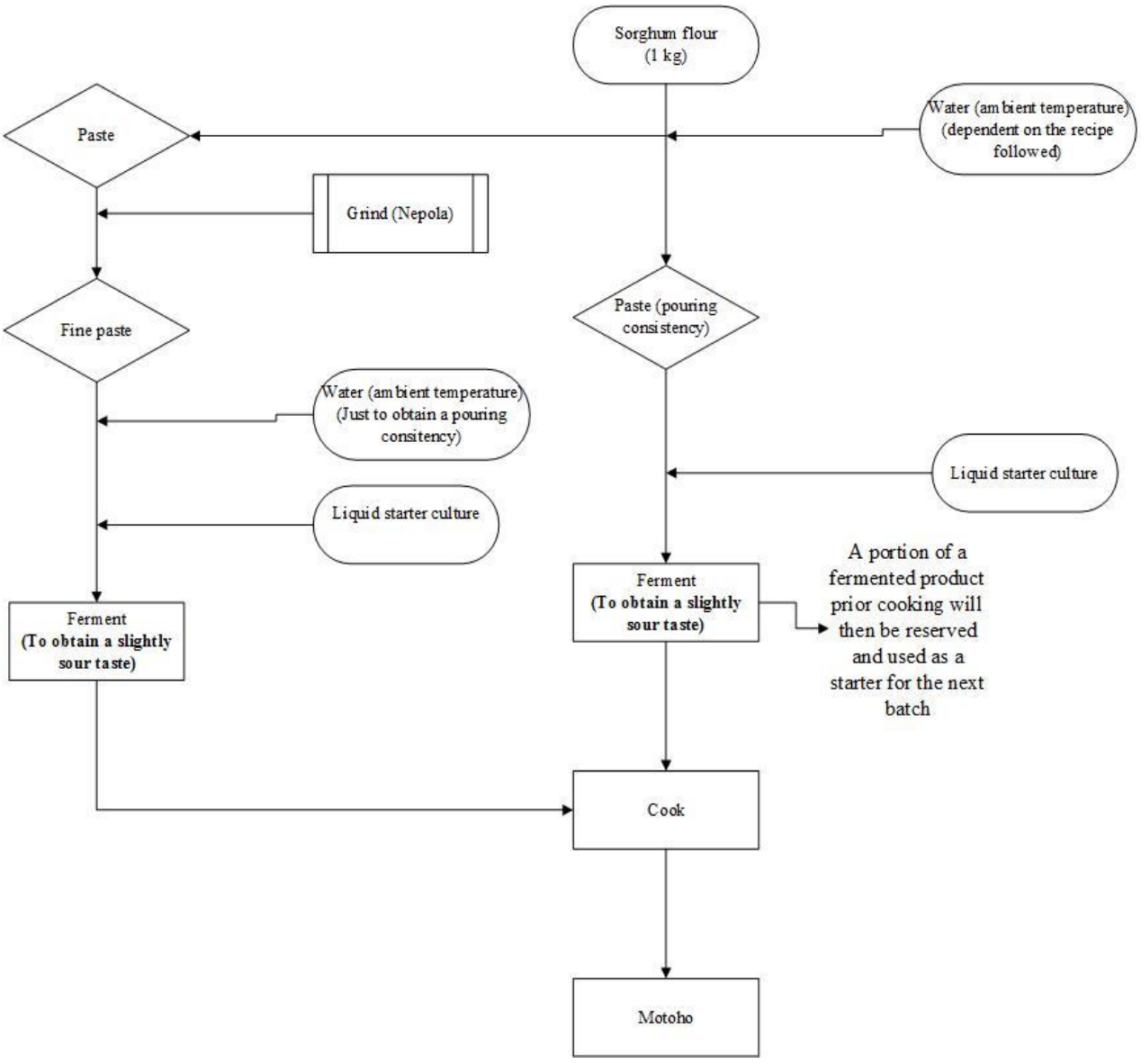

Figure 2.6: A flow diagram illustrating *motoho* preparation (~15 L).

Figure 2.7: *Motoho* on the market shelves in Lesotho.

2.7.2.3 Hopose

Hopose is another popular traditional alcoholic fermented beverage in Lesotho. It is produced from wheat flour. It is turbid and has a thin consistency. Its name is derived from the ingredient 'hops' (usually purchased from local markets), which is an important ingredient contributing to its bitter taste. *Hopose* is prepared at house-hold level as an inebriating drink at marriages, traditional ceremonies as well as being prepared for commercial purposes at village level.

Hopose preparation entails mixing wheat flour with cold water to form a paste (fig 2.8). Boiling water is then added to the mixture to form a thin paste. The mixture is then cooled. Brewer's yeast (purchased from local markets) or *moroko* is added to the cooled paste. In a separate container, hops is boiled with a bit of wheat flour until the mixture attains a green colour. Then the hops mixture and sugar are added to the wheat flour mix followed by dilution of the mixture with cold water and addition of *mmela* and *moroko*. The mixture is left overnight to ferment. After fermentation, the mixture is filtered to get rid of dregs prior to consumption.

From the information obtained, *hopose* is perceived as a modernised cereal based alcoholic beverage by the usual traditional beer consumers as it uses hops, and a commercial brewer's yeast purchased from local markets. Thus, *hopose* is not common in the rural areas of Lesotho and it is mostly preferred in the urban areas.

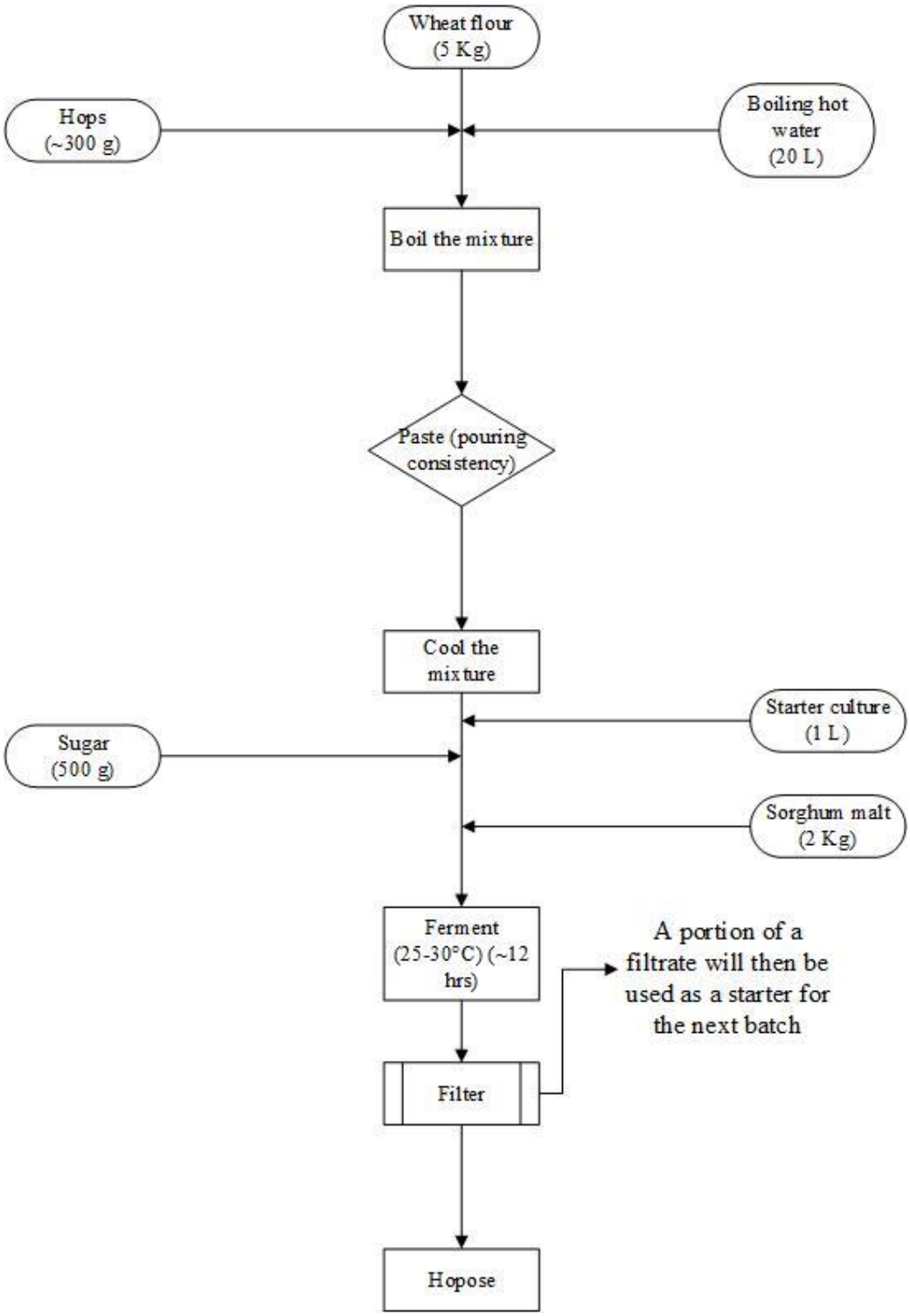

Figure 2.8: A flow diagram illustrating *hopose* preparation (~15 L).

2.7.2.4 Tsoeu-koto

Tsoeu-koto is a common traditional fermented non-alcoholic gruel prepared in Lesotho. Can be slightly alcoholic depending on its age, as the final product undergoes spontaneous fermentation. This gruel is produced from white-type maize, which explains its name *tsoeu,* a term for white (sesotho language); this is by virtue of the white-type maize used in its production, thus giving this gruel a white appearance. This non-alcoholic gruel is known by different names such as *mahleu* and *tsoeu poone* at different localities. *Tsoeu-koto* is consumed widely by adults at village-level as a thirst quenching gruel. It is not commonly served at cultural ceremonies.

Despite different names at different localities, the preparation of *tsoeu koto* is the same throughout the country. It comprises of a series of operations; white maize flour is mixed with water (ambient temperature), the paste is then added into boiling water and cooked until a thinner smooth paste is formed. After cooking, the mixture is allowed to cool; *tomoso* (*mmela* or *moroko*) is added to the cooled mixture. The brewed mixture is covered and left to ferment overnight prior consumption (fig 2.9).

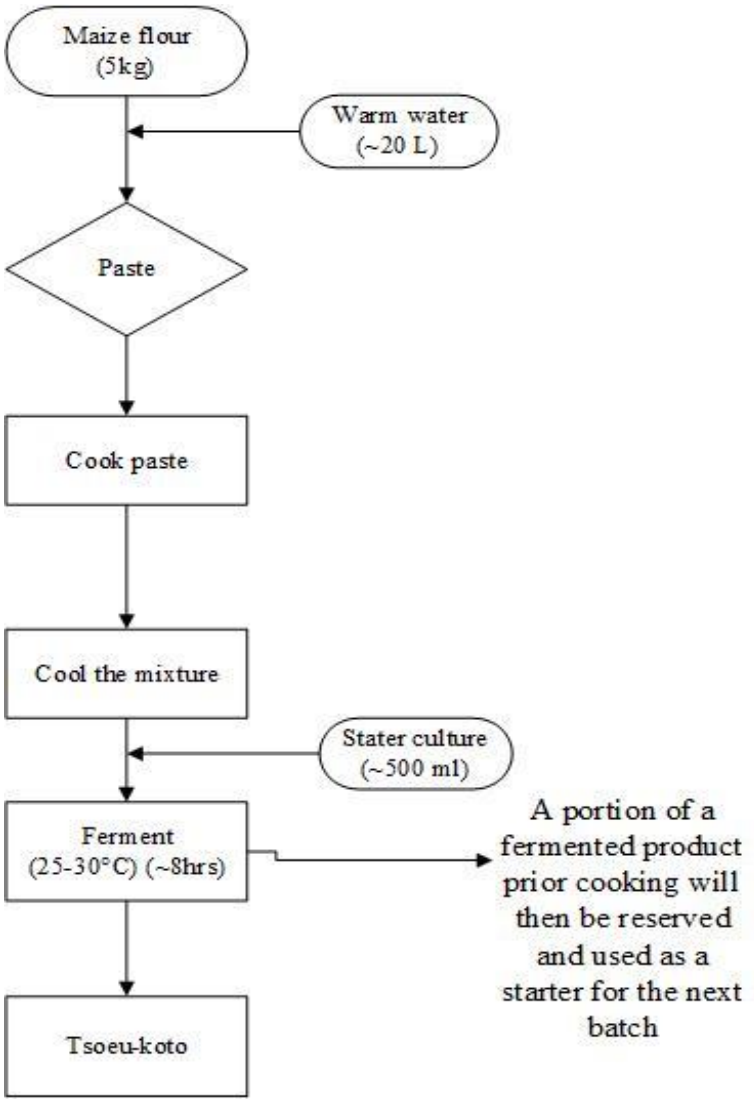

Figure 2.9: A flow diagram illustrating *tsoeu-koto* preparation (~15 L).

2.7.2.5 Tjontjobina

Tjontjobina otherwise famously known as *sekumukumu*, is produced from residues of either sorghum bread or maize meal pap or otherwise a mixture of both. *Sekukumuku* is a general term used to explain the fermentation of residuals from cereal produced foods. This traditional beer is widely consumed at village-level as an inebriating drink at cultural ceremonies and it is not common for small scale commercial purposes.

Its preparation involves mixing residues of sorghum bread or pap/maize meal pap with warm water and cooking to form a paste. The paste is cooled and *tomoso* added to the paste, the mixture is then left to ferment overnight. Following fermentation, the mixture is filtered prior to consumption (fig. 2.10).

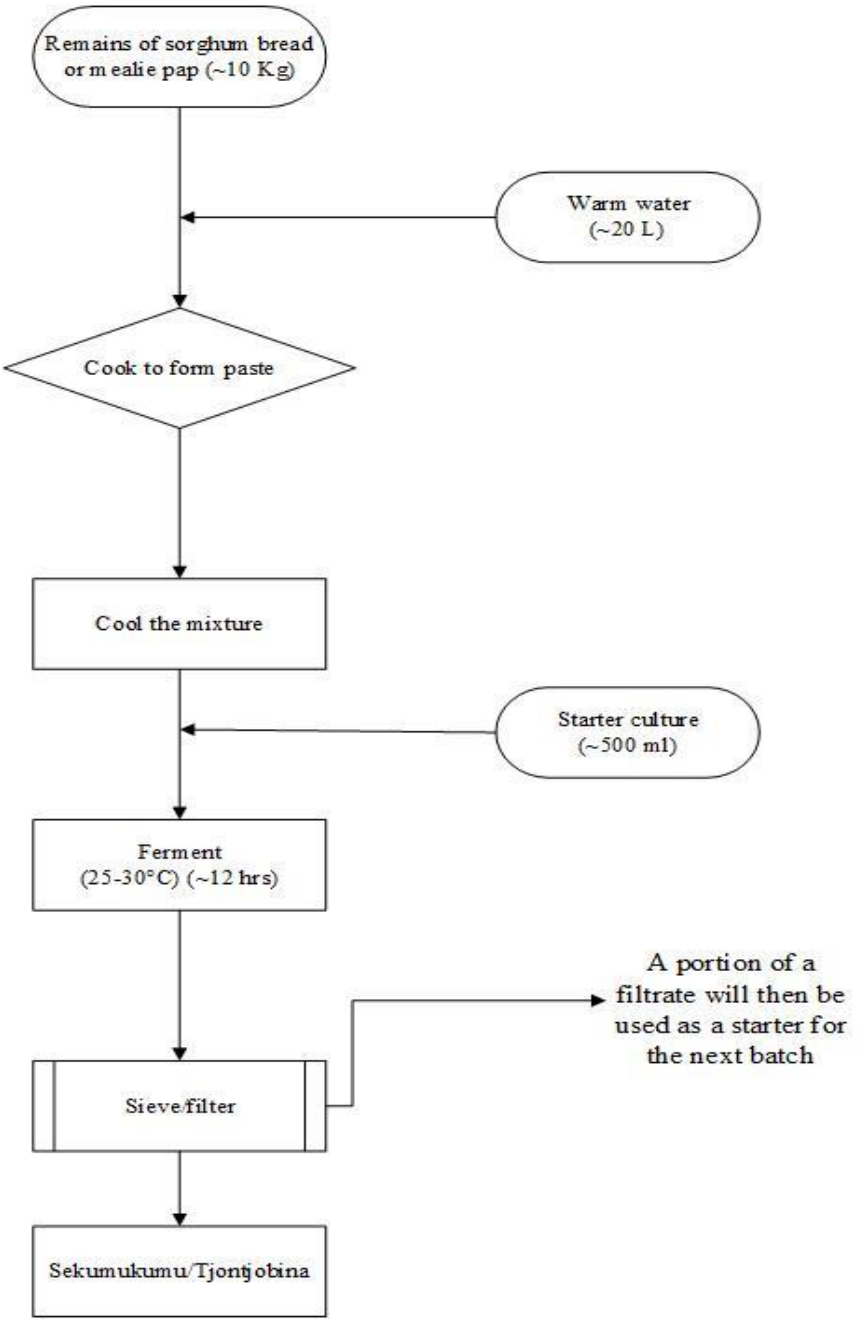

Figure 2.10: A flow diagram illustrating *sekumukumu* preparation (~ 15 L).

2.7.2.6 Ntsoana-tsike

Ntsoana-tsike is a fermented sorghum bread, otherwise popularly known as *papa ea mabele*. It was commonly consumed at house-hold level in rural areas by adults during bridal acquisition as well as during the bridal reception. It is however not commonly used for commercial purposes. *Ntsoana-tsike* preparation entails mixing sorghum flour with warm water to form dough, and then mixing in m*oroko*. The dough is then covered and allowed to ferment for about 3-4 hours. Following fermentation, the dough is

modelled into small dough balls which are steamed prior to consumption (fig. 2.11). The preparation of *ntsoana-tsike* is not common in the urban areas. This is mainly because most urban citizens have slightly shifted away from the traditional practices.

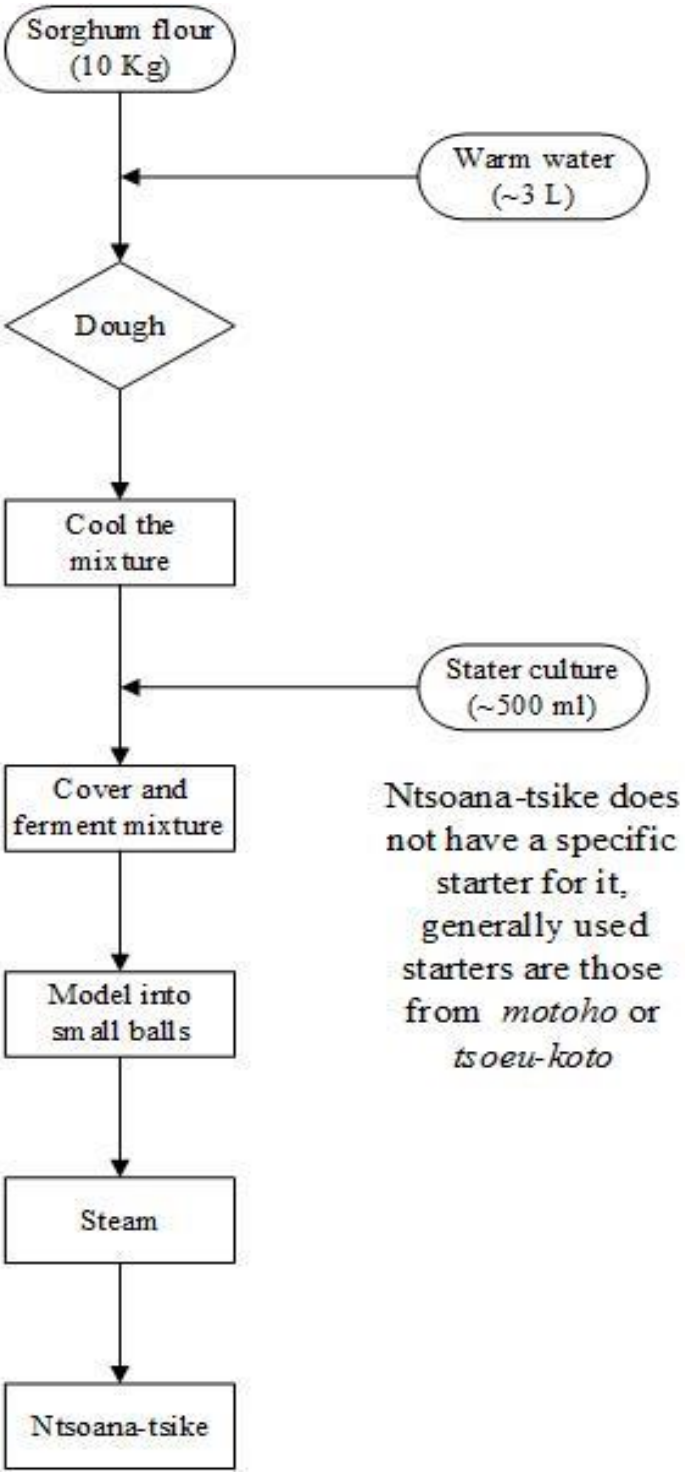

Figure 2.11: A flow diagram illustrating *ntsoana-tsike* preparation (~20 balls).

2.7.2.7 Tintana

Tintana is an ancient fermented alcoholic beverage in Lesotho. Oral history from senior citizens provided that this beverage was produced from roasted sorghum and therefore had a dark appearance. Its preparation is an indigenous knowledge that got lost from generation to generation during the process of time as it was labour intensive (with reference to sorghum germination and dough roasting) and complicated. The preparation of *tintana* comprises of a series of steps; roasted dough made from geminated sorghum is mixed with hot water, the mixture is allowed to cool, then *moroko* is added (fig. 2.12). The mixture is then allowed to ferment overnight; the mixture would produce large bubbles in the last stages of fermentation. After fermentation, the mixture is filtered to get rid of the dregs, and then cooked for about 2 hours before consumption.

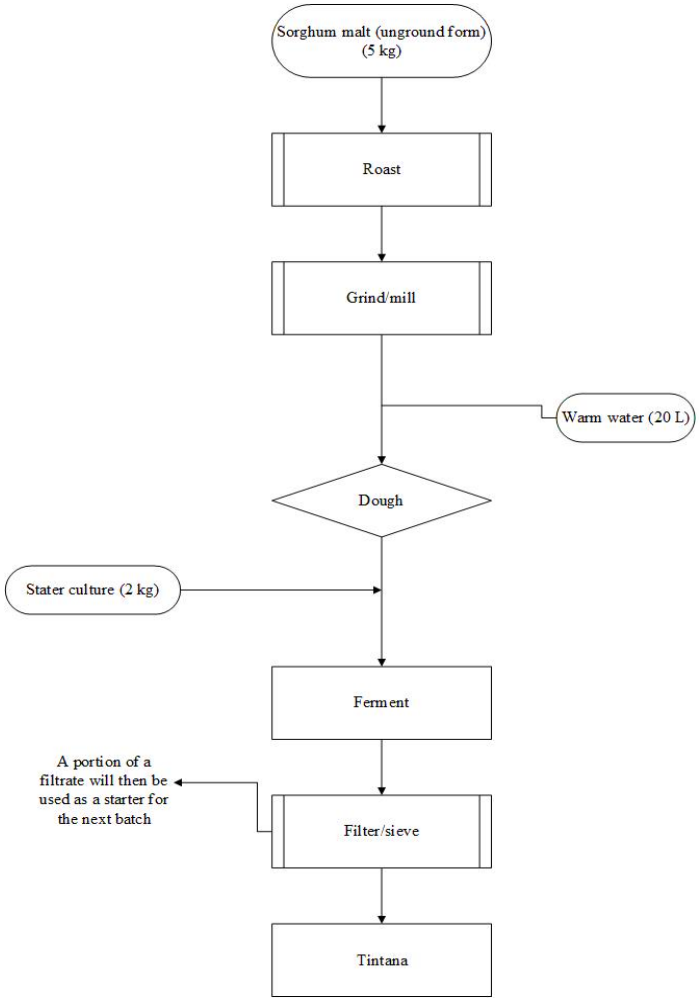

Figure 2.12: A flow diagram illustrating *tintana* preparation (~ 15 L).

2.8 Benefits of cereal fermentation

Cereals are of great importance, particularly in Africa where they are utilized as staples as well as substrates for fermented food products (Nout, 2009). Cereal fermentation brings about desirable food modifications in food quality as well as characteristic properties such as flavour, aroma and texture (Abee *et al.*, 1995; Achi & Ukwuru, 2015; Awika & Rooney, 2004; Blandino *et al.*, 2003). Apart from improving the palatability and consumer acceptability through developing improved flavours and textures, fermentation also preserves food through the formation of alcohol, acidulants and antimicrobial compounds (Caplice & Fitzgerald, 1999). Fermentation also improves the nutritive content of fermented products by microbial synthesis of essential nutrients and improving digestibility of carbohydrates, essential amino acids, vitamins and trace elements. Extensive studies on the safety of fermented foods have also shown that fermentation reduces anti-nutritive compounds, mycotoxins and natural toxicants that are naturally present on the cereals (Hammes *et al.*, 2005). Fermentation of cereals also reduces the cooking times (Steinkraus, 1997).

2.9 Nutritional quality of cereal based fermented foods

Fermented foods are major contributors to the diet in developing countries. Cereals in particular are important substrates for fermented foods in these parts of the world. However, cereals and other plant foods may contain significant amounts of toxic or anti-nutritional substances that reduce the bioavailability of minerals such as iron, zinc, magnesium and calcium as well as contributing to the deficiency of essential amino acids. Most cereals contain appreciable amounts of phytates, saponins and enzyme inhibitors. Some cereals such as millets and sorghum contain large amounts of polyphenols and tannins (Awika & Rooney, 2004; Benito & Miller, 1998; El Hag *et al.*, 2002; Tapiero *et al.*, 2001). The presence of these anti-nutrients has negative effects on the content of the nutritional quality of the food product hence the need for their reduction or removal. However, fermentation of cereals, fruits and vegetables can transform otherwise inedible food supplies to edible, safe, nutritious food stuffs as well as enhancing digestibility of carbohydrates, proteins and eliminating toxic substances (Chelule *et al.*, 2010).

2.9.1 Fermentation and anti-nutritional compounds

2.9.1.1 Phytate

Phytic acid is found within the hulls of nuts, grains and seeds. Phytic acid reduces the bioavailabilty of minerals, the solubility, functionality and the digestibility of proteins and carbohydrates. Fermentation of cereals reduces phytate content through phytases that catalyse the conversion of phytic acid to inorganic orthophosphate as well as a series of myoinositols, lower phosphoric esters of phytic acid (Elyas *et al.*, 2002; El Hag *et al.*, 2002).

2.9.1.2 Tannins

Tannins occur in cereals and legumes. Tannin-protein complexes inactivate the digestive enzymes, reducing the protein digestibility through the interaction of protein substrate with ionised iron (Awika & Rooney, 2004; Dykes & Rooney, 2006; Elyas *et al.*, 2002). Presence of tannins in food lower the feed efficiency, decrease iron absorption, retard growth, decrease iron absorption, damage the mucosal lining of the gastrointestinal tract and increase the excretion of proteins and those essential amino acids. Operations such as dehulling, cooking as well as fermentation reduce the tannin content of cereals. Reduction in tannin levels leads to increased absorption of iron (Dlamini *et al.*, 2007; Marshall & Mejia-Lorio, 2012).

2.9.1.3 Enzyme inhibitors

Fermentation also reduces the enzyme inhibitors. Enzymes such as proteases and amylases are found in seed and grain tissues. Enzyme inhibitors such as trypsin and chymotrypsin as well as the cysteine protease inhibitors are believed to cause growth retardation through interfering with digestion, causing pancreatic hypertrophy and disturbing the utilisation of sulphur amino acid (Blandino *et al.*, 2003; Hammes *et al.*, 2005; Motarjemi, 2002).

2.10 Hygiene and safety risks associated with lactic acid fermentation

Fermented foods generally have a good safety record, even in rural areas or developing countries where the producers have little or no knowledge on the hygienic practices (Curtis *et al.*, 2011; Steinkraus, 2002). This is because the fermentation process itself inhibits the growth and survival of several pathogenic bacteria. However,

the bacterial inhibition depends on the organism concerned, the temperature and the amount of undissociated acid produced in food i.e. buffering capacity. Lactic acid fermentation of cereals and vegetables will produce a pH level of 4 or less. These conditions inhibit the pathogenic bacteria, but then in these acidic conditions many bacteria including those that are beneficial will die (Motarjemi & Nout, 1996; Steinkraus, 2002, 1997).

High post-harvest losses that arise from limited food preservation capacity are the main contributing factors to food nutrition and security in developing countries, where seasonal food shortages as well as nutritional deficiency diseases are still of great concern. Various micronutrient deficiency disorders such as vitamin A deficiencies, nutritional anaemias due to lack or shortage of folic acid, iron and vitamin B_{12} continue to present important health concerns in developing countries. Protein-energy malnutrition and iron deficiency disorders have retardation effects in growth as well as mental development of children (Bassey *et al.*, 2013; Franz *et al.*, 2014; Motarjemi & Nout, 1996; Svanberg & Lorri, 1997). These deficiency disorders are major contributors to the high rates of child and maternal morbidity and mortality. Inappropriate or ineffective processing methods, careless harvesting practices as well as lack of storage facilities are also some contributing factors to high post-harvest food losses in developing countries (Fnifst, 2008).

It is of great importance to note that fermentation itself does not replace the need for hygienic practices. Contamination of fermented foods depends on several factors that are difficult to quantify such as; initial level of contamination on raw materials, levels of hygiene and sanitation (Benkerroum, 2013; Nout & Ngoddy, 1997; Steinkraus, 1997). The degree of acidification is important as some pathogens are acid-tolerant and thus survive the fermentation process. Examples of such microorganisms are *Escherichia coli* O157:H7 and foodborne rotavirus that causes childhood diarrhoea (Cálix-Lara *et al.*, 2014; Motarjemi, 2002).

Lactic acid produced from microbial activity can be a mixture of optical isomers of **L**-(-) and **D**-(-). The latter cannot be metabolised by humans and excessive intake has been reported to cause acidosis (Motarjemi & Nout, 1996). Potential toxicity of **D**-(-) lactic acid for malnourished and sick children is of great concern, thus further

research regarding its occurrence in foods prepared at household level is required (Rolle & Satin, 2002).

2.10.1 Mycotoxins

Mycotoxins are secondary metabolites produced by fungi. These metabolites comprise of a wide range of molecules that have harmful effects to humans as well as to animals. Their contamination is dependent on the production facilities as well as on the storage conditions. Mycotoxins have varied chemical compositions, as well as different health effects. Several studies have reported mycotoxins to have tetratogenic, haemorrhagic, carcinogenic and dermatitic effects on a diverse range of organisms as well as causing hepatic carcinoma in humans (Fandohan *et al.*, 2005; Juodeikiene *et al.*, 2012; Kpodo *et al.*, 1996).

Many fermented food products (e.g. Blue cheese) are prepared with the use of fungal species and other food products (e.g. *Mahewu*) make use of substrates (e.g. Cereals) that are subject to fungal contamination upon poor storage conditions or sometimes as a result of field contamination. As result, the risk of mycotoxin contamination is quite high in fermented foods. . The majority of these toxic organic molecules are produced by *Penicillium* and *Aspergillus* genera, nonetheless, some studies have indicated that *Fusarium* species also produce some potent toxins as well (Kpodo *et al.*, 1996).

Of all the mycotoxins, aflatoxins have been extensively studied. Aflatoxins are produced by *Aspergillus parasiticus* and *Arspergillus flavus* species which are widely distributed. Concerns whether mycotoxins are associated with traditional fermented foods have been raised since the discovery of aflatoxins. This was confirmed as aflatoxins were detected in some indigenous fermented foods (Westby *et al.*, 1997). Mycotoxin producing fungi found in fermented food products originate from contaminated raw materials, poor storage conditions, and poor sanitary practices or from contamination during fermentation. As a result, the presence of toxinogenic fungi in fermented food products is thus of concern as it increases the risk of mycotoxins in these foods (Fandohan *et al.*, 2005; Kpodo *et al.*, 1996).

Despite the risk of mycotoxins associated with fermented food products, several studies have been done on the fate of fumonisins and aflatoxins during the preparation of fermented food products (Fandohan *et al.*, 2005). Some operations during the

preparation of these fermented food products have been found to significantly reduce the mycotoxin. The critical steps in mycotoxin reduction have been found to be cleaning, sorting and washing of the fermented cereal substrates. Fandohan and co-workers in 2005 demonstrated that up to 91% of the mycotoxins are reduced during these stages. Cleaning and washing of the grains was not negligible, as significant quantities of aflatoxins and fumonisins were seen in washing water. Nonetheless, hand-sorting visibly mouldy grains with the aim to reduce the mycotoxin levels are likely to depend on the ability of the people responsible in carrying out the operation. As such, people who are well trained to recognise the defected grains are thus more likely to carry out the operation more effectively (Fandohan *et al.*, 2005).

2.11 Future of African cereal based fermented foods and concluding remarks

Fermented foods generally have a good safety record as they contain some antimicrobial factors to inhibit pathogenic microorganisms and other undesirable microorganisms which may generally spoil the fermented product (Banu *et al.*, 2012; Campbell-Platt, 1994; Steinkraus, 1994, 1997). Fermented food products are very likely candidates to remain an integral part as food supply for human particularly in Africa. It is thus of great importance that African citizens are educated on the importance of consuming fermented food products and their health benefits. However, there are several drawbacks regarding fermented products as they lack standardised preparation methods, prepared under unhygienic conditions thus leading to products with poor quality, a shorter shelf-life and potentially put the lives of those consuming them in danger (Blandino *et al.*, 2003; Curtis *et al.*, 2011; Guyot, 2012; Mensah, 1997; Motarjemi, 2002; Rolle & Satin, 2002). The other drawback is that traditional fermented products lack appeal in presentation and marketing as they are not packaged in an attractive manner with proper labelling. Extensive research on fermentation as a house-hold technology is needed so as to advance its potential for nutritional value and food safety. Improvement of the technology to ensure that fermented products have longer shelf-life, nutritionally stable, safer, have an appealing packaging and labelling remains to be the greatest challenge (Chelule *et al.*, 2010).

The microbial composition of the fermentation processes are of paramount importance as they contribute greatly to the safety, taste, texture, aroma as well as the nutritional benefits of the final product (Chelule *et al.*, 2010; Mokoena *et al.*, 2016). Specific

starter cultures for traditional fermented products are not yet available on the market. It is very important that the traditional starter cultures are developed as research has shown the importance of predominant bacteria in the lactic fermentation, particularly LAB.

Documentation of these indigenous traditional fermented products is also important as it will conserve this vital indigenous knowledge for future generations of historians, microbiologists, biochemists and food scientists. The microbial and biochemical assessments of these traditionally fermented products are thus of paramount importance and these studies will serve as a crucial stepping stone towards the development of the starter cultures with valuable health benefits as well as enabling the predictability of safe fermented products (da Silva Sabo *et al.*, 2014). It will also enable the creation of a reference database for food scientists, nutritionists, historians as well as the policy makers in governments.

2.12 References

Abee, T., Krockel, L. & Hill, C. (1995). Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. *Int J Food Microbiol* **28**, 169–185.

Achi, O. K. & Ukwuru, M. (2015). Cereal-Based Fermented Foods of Africa as Functional Foods. *Int J Microbiol Appl* **2**, 71–83.

Ahmad, V., Khan, M. S., Jamal, Q. M. S., Alzohairy, M. A., Al Karaawi, M. A. & Siddiqui, M. U. (2016). Antimicrobial potential of bacteriocins: in therapy, agriculture and food preservation. *Int J Antimicrob Agents* **49**, 1–11.

Ali, A. A. & Mustafa, M. . (2009). Use of starter cultures of lactic acid bacteria and yeasts in the preparation of kirsa, a sudanese fermented food. *Pakistan J Nutr* **9**, 1349–1353.

Amenan Anastasie Soro-Yao, Kouakou Brou, Georges Amani, P. T. and K. M. D. (2014). The use of LAB in starter cultures during the processing of fermented cereal based foods in west africa: a review. *Trop Life Sci Res* **25**, 1–10.

Anukam, K. C. & Reid, G. (2009). African Traditional Fermented Foods and Probiotics. *J Med Food* **12**, 1177–1184.

Assohoun-Djeni, N. M. C., Djeni, N. T., Messaoudi, S., Lhomme, E., Koussemon-Camara, M., Ouassa, T., Chobert, J. M., Onno, B. & Dousset, X. (2016). Biodiversity, dynamics and antimicrobial activity of lactic acid bacteria involved in the fermentation of maize flour for doklu production in Cote d'Ivoire. *Food Control* **62**, 397–404.

Awika, J. M. & Rooney, L. W. (2004). Sorghum phytochemicals and their potential impact on human health. *Phytochemistry* **65**, 1199–1221.

Banu, H., Itagi, N., Jayadeep, A. & Singh, V. (2012). Nutrients , Nutraceuticals and Bioactive Properties of Multi-Whole Grain Mix for Drink and Porridge. *J nutrtion Ther* **1**, 161–171.

Bassey, F. I., McWatters, K. H., Edem, C. a & Iwegbue, C. M. a. **(2013).** Formulation and nutritional evaluation of weaning food processed from cooking banana, supplemented with cowpea and peanut. *Food Sci Nutr* **1**, 384–91.

Benito, P. & Miller, D. (1998). Iron absorption and bioavailability: An updated review. *Nutr Res* **18**, 581–603.

Benkerroum, N. (2013). Traditional Fermented Foods of North African Countries : Technology and Food Safety Challenges With Regard to Microbiological Risks. *Compr Rev food Sci food Saf* **12**, 54–89.

Blandino, A., Al-Aseeri, M. E., Pandiella, S. S., Cantero, D. & Webb, C. (2003). Cereal-based fermented foods and beverages. *Food Res Int* **36**, 527–543.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., Harnett, J., Huys, G., Laulund, S. & other authors. **(2012).** Food fermentations: Microorganisms with technological beneficial use. *Int J Food Microbiol* **154**, 87–97.

Cálix-Lara, T. F., Rajendran, M., Talcott, S. T., Smith, S. B., Miller, R. K., Castillo, A., Sturino, J. M. & Taylor, T. M. (2014). Inhibition of Escherichia coli O157: H7 and Salmonella enterica on spinach and identification of antimicrobial substances produced by a commercial Lactic Acid Bacteria food safety intervention. *Food Microbiol* **38**, 192–200.

Campbell-Platt, G. (1994). Fermented foods — a world perspective. *Food Res Int* **27**, 253–257.

Caplice, E. & Fitzgerald, G. F. (1999). Food fermentations: Role of microorganisms in food production and preservation. *Int J Food Microbiol* **50**, 131–149.

Chelule, P., Mokoena, M. & Gqaleni, N. (2010). Advantages of traditional lactic acid bacteria fermentation of food in Africa. *Curr Res Technol Educ Top Appl Microbiol Microb Biotechnol* **2**, 1160–1167.

Curtis, V., Schmidt, W., Luby, S., Florez, R., Touré, O. & Biran, A. (2011). Hygiene: new hopes, new horizons. *Lancet Infect Dis* **11**, 312–21.

Deegan, L. H., Cotter, P. D., Hill, C. & Ross, P. (2006). Bacteriocins: Biological tools for bio-preservation and shelf-life extension. *Int Dairy J* **16**, 1058–1071.

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

Dykes, L. & Rooney, L. W. (2006). Sorghum and millet phenols and antioxidants. *J Cereal Sci* **44**, 236–251.

Elyas, S. H. A., El Tinay, A. H., Yousif, N. E. & Elsheikh, E. A. E. (2002). Effect of natural fermentation on nutritive value and in vitro protein digestibility of pearl millet. *Food Chem* **78**, 75–79.

Fandohan, P., Zoumenou, D., Hounhouigan, D. J., Marasas, W. F. O., Wingfield, M. J. & Hell, K. (2005). Fate of aflatoxins and fumonisins during the processing of maize into food products in Benin. *Int J Food Microbiol* **98**, 249–259.

Fnifst, O. (2008). *The Role of Traditional Food Processing Technologies In National Development: the West African Experience*. University of Ibadan.

Franz, C. M. A. P., Huch, M., Maina, J., Abriouel, H., Benomar, N., Reid, G., Galvez, A. & Holzapfel, W. H. (2014). International Journal of Food Microbiology African fermented foods and probiotics. *Int J Food Microbiol* **190**, 84–96.

Gadaga, T. H., Lehohla, M. & Ntuli, V. (2013). Traditional Fermented Foods of Lesotho. *J Microbiol Biotechnol Food Sci* **2**, 2387–2391.

Giraffa, G. (2004). Studying the dynamics of microbial populations during food fermentation. *FEMS Microbiol Rev* **28**, 251–260.

Greppi, A., Rantisou, K., Padonou, W., Hounhouigan, J., Jespersen, L., Jakobsen, M. & Cocolin, L. (2013). Yeast dynamics during spontaneous fermentation of mawe and tchoukoutou, two traditional products from Benin. *Int J Food Microbiol* **165**, 200–207.

Guyot, J.-P. (2012). Cereal-based fermented foods in developing countries: ancient foods for modern research. *Int J Food Sci Technol* **47**, 1109–1114.

El Hag, M. E., El Tinay, A. H. & Yousif, N. E. (2002). Effect of fermentation and dehulling on starch, total polyphenols, phytic acid content and in vitro protein digestibility of pearl millet. *Food Chem* **77**, 193–196.

Hammes, W. P., Brandt, M. J., Francis, K. L., Rosenheim, J., Seitter, M. F. H. & Vogelmann, S. A. (2005). Microbial ecology of cereal fermentations. *Trends Food Sci Technol* **16**, 4–11.

Holzapfel, W. H. (2002). Appropriate starter culture technologies for small-scale fermentation in developing countries. *Int J Food Microbiol* **75**, 197–212.

Holzapfel, W. (1997). Use of starter cultures in fermentation on a household scale. *Food Control* **8**, 241–258.

Iacumin, L., Chiesa, L., Boscolo, D., Manzano, M., Cantoni, C., Orlic, S. & Comi, G. (2009). Moulds and ochratoxin A on surfaces of artisanal and industrial dry sausages. *Food Microbiol* **26**, 65–70.

Ijabadeniyi, a O. (2007). Microbiological safety of gari , lafun and ogiri in Akure metropolis , Nigeria. *J Biotechnol* **6**, 2633–2635.

Juodeikiene, G., Basinskiene, L., Bartkiene, E. & Matusevicius, P. (2012). Mycotoxin Decontamination Aspects in Food , Feed and Renewables Using Fermentation Processes. In *Struct Funct food Eng*, pp. 171–204.

Kandler, O. (1983). Carbohydrate metabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek* **49**, 209–224.

Katongole, J. (2008). *The Microbial Succession in Indigenous Fermented Maize Products*. University of the Free State.

Kayodé, a P. P., Linnemann, a R. & Kotchoni, S. O. (2011). Diversity of yeasts involved in the fermentation of tchoukoutou , an opaque sorghum beer from Benin. *J Microbiol* **5**, 2737–2742.

Khalid, K. (2011). An overview of lactic acid bacteria. *Int J Biosci* **1**, 2222–5234.

Kingamkono, R., Sjogren, E., Svanberg, U. & Kaijser, B. (1997). pH and acidity in lactic-fermenting cereal gruels: Effects on viability of enteropathogenic microorganisms. *Doktorsavhandlingar vid Chalmers Tek Hogsk* **10**, 664–669.

Kpodo, K., Sørensen, a. K. & Jakobsen, M. (1996). The occurrence of mycotoxins in fermented maize products. *Food Chem* **56**, 147–153.

Kuye, A. & Sanni, L. O. (1999). Industrialization of fermented food processes: How far in Nigeria? *J Sci Ind Res (India)* **58**, 837–843.

Lin, X. B. & Gänzle, M. G. (2014). Quantitative high-resolution melting PCR analysis for monitoring of fermentation microbiota in sourdough. *Int J Food Microbiol* **186**, 42–48.

MacDonald, A., Evans, S., Cochrane, B. & Wildgoose, J. (2012). Weaning infants with phenylketonuria: a review. *J Hum Nutr Diet* **25**, 103–10.

Majara, N. (2005). *Land Degradation in Lesotho : A Synoptic perspective*. University of Stellenbosch.

Marco, M. L., Heeney, D., Binda, S., Cifelli, C. J., Cotter, P. D., Foligné, B., Gänzle, M., Kort, R., Pasin, G. & other authors. **(2017).** Health benefits of fermented foods : microbiota and beyond Michael Ga. *Curr Opin Biotechnol* **44,** 94–102.

Marsh, A. J., Hill, C., Ross, R. P. & Cotter, P. D. (2014). Fermented beverages with potential : Past and future perspectives. *Trends Food Sci Technol* **38**, 113–124.

Marshall, E. & Mejia-Lorio, D. J. (2012). Traditional fermented food and beverages for improved livelihoods. *Diversif Bookl*.

Mensah, P. (1997). Fermentation — the key to food safety assurance in Africa? *Food Control* **8**, 271–278.

Mokoena, M. P., Mutanda, T. & Olaniran, A. O. (2016). African traditional fermented foods and beverages. *Food Nutr Res* **1**, 1–12.

Motarjemi, Y. & Nout, M. J. (1996). Food fermentation: a safety and nutritional assessment. Joint FAO/WHO Workshop on Assessment of Fermentation as a Household Technology for Improving Food Safety. In *Bull World Health Organ*, pp. 553–559.

Motarjemi, Y. (2002). Impact of small scale fermentation technology on food safety in developing countries. *Int J Food Microbiol* **75**, 213–229.

Mufandaedza, J., Viljoen, B. C., Feresu, S. B. & Gadaga, T. H. (2006). Antimicrobial properties of lactic acid bacteria and yeast-LAB cultures isolated from traditional fermented milk against pathogenic Escherichia coli and Salmonella enteritidis strains. *Int J Food microbiol* **108**, 147–152.

Mugula, J. K., Nnko, S. a M., Narvhus, J. a. & Sørhaug, T. (2003). Microbiological and fermentation characteristics of togwa, a Tanzanian fermented food. *Int J Food Microbiol* **80**, 187–199.

Muller, V. (2003). MINIREVIEW Energy Conservation in Acetogenic Bacteria. *Appl Environ Microbiol* **69**, 6345–6353.

Muyanja, C. M. B. K., Narvhus, J. A., Treimo, J. & Langsrud, T. (2003). Isolation, characterisation and identification of lactic acid bacteria from bushera: A Ugandan traditional fermented beverage. *Int J Food Microbiol* **80**, 201–210.

Narvhus, J. A. & Henry, T. (2003). The role of interaction between yeasts and lactic acid bacteria in African fermented milks : a review **86**, 51–60.

Nout, M. J. R. (2009). Rich nutrition from the poorest - cereal fermentations in Africa and Asia. *Food Microbiol* **26**, 685–692.

Nout, M. J. R. & Ngoddy, P. O. (1997). Technological aspects of preparing affordable fermented complementary foods. *Food Control* **8**, 279–287.

Nout, M. J. R., Kok, B., Vela, E., Nche, P. F. & Rombouts, F. M. (1995). Acceleration of the fermentation of kenkey, an indigenous fermented maize food of Ghana. *Food Res Int* **28**, 599–604.

Odunfa, S. a. (1988). Review: African fermented foods: from art to science. *MIRCEN J* **4**, 259–273.

Oyedeji, O., Ogunbanwo, S. T. & Onilude, A. A. (2013). Predominant Lactic Acid Bacteria Involved in the Traditional Fermentation of Fufu and Ogi, Two Nigerian Fermented Food Products. *Food Nutr Sci* **4**, 40–46.

Rakin, M., Vukasinovic, M., Siler-Marinkovic, S. & Maksimovic, M. (2007). Contribution of lactic acid fermentation to improved nutritive quality vegetable juices enriched with brewer's yeast autolysate. *Food Chem* **100**, 599–602.

Rhee, S., Lee, J.-E. & Lee, C.-H. (2011). Importance of lactic acid bacteria in Asian fermented foods. *Microb Cell Fact* **10**, S5.

Rolle, R. & Satin, M. (2002). Basic requirements for the transfer of fermentation technologies to developing countries. *Int J Food Microbiol* **75**, 181–187.

Schillinger, U., Geisen, R. & Holzapfel, W. H. (1996). Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. *Trends Food Sci Technol* **7**, 158–164.

Selhub, E. M., Logan, A. C. & Bested, A. C. (2014). Fermented foods, microbiota, and mental health: ancient practice meets nutritional psychiatry. *J Physiol Anthropol* **33**, 2.

da Silva Sabo, S., Vitolo, M., González, J. M. D. & Oliveira, R. P. de S. (2014). Overview of Lactobacillus plantarum as a promising bacteriocin producer among lactic acid bacteria. *Food Res Int* **64**, 527–536.

Simango, C. (1997). Potential use of traditional fermented foods for weaning in Zimbabwe. *Soc Sci Med* **44**, 1065–1068.

Smid, E. J. & Lacroix, C. (2013). Microbe-microbe interactions in mixed culture food fermentations. *Curr Opin Biotechnol* **24**, 148–154.

Steinkraus, H. K. (2002). Fermentations in world food processing. *Compr Rev food Sci food Saf* **1**, 23–32.

Steinkraus, K. H. (1983). Fermented foods, feeds and beverages. *Biotechnol Adv* **1**, 31–46.

Steinkraus, K. H. (1994). Nutritional significance of fermented foods. *Food Res Int* **27**, 259–267.

Steinkraus, K. H. (1997). Classification of fermented foods: worldwide review of household fermentation techniques. *Food Control* **8**, 311–317.

Svanberg, U. & Lorri, W. (1997). Fermentation and nutrient availability. *Food Control* **8**, 319–327.

Taiwo, O. (2009). Physical and nutritive properties of fermented cereal foods **3**, 23–27.

Tamang, J. P., Watanabe, K. & Holzapfel, W. H. (2016). Review : Diversity of Microorganisms in Global Fermented Foods and Beverages. *Front Microbiol* **7**, 377.

Tapiero, H., Gaté, L. & Tew, K. D. (2001). Iron: Deficiencies and requirements. *Biomed Pharmacother* **55**, 324–332.

Todorov, S. D. & Holzapfel, W. H. (2014). Traditional cereal fermented foods as sources of functional microorganisms. In *Adv Fermented Foods Beverages Improv Qual Technol Heal Benefits*, pp. 123–148. Edited by W. Holzapfel.

Vuyst, L. De. **(2004).** Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci Technol* **15**, 67–78.

Watanabe, K., Fujimoto, Æ. J., Sasamoto, Æ. M. & Dugersuren, Æ. J. (2008). uctDiversity of lactic acid bacteria and yeasts in Airag and Tarag , traditional fermented milk prods of Mongolia. *World J Microbiol Biotechnol* **24**, 1313–1325.

Westby, A., Reilly, A. & Bainbridge, Z. (1997). Review of the effect of fermentation on naturally occurring toxins. *Food Control* **8**, 329–339.

Yang, E., Fan, L., Jiang, Y., Doucette, C. & Fillmore, S. (2012). Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. *AMB Express* **2**, 48.

Zorba, M., Hancioglu, O., Genc, M., Karapinar, M. & Gulden, O. (2003). The use of starter cultures in the fermentation of boza , a traditional Turkish be v erage. *Process Biochem* **38**, 1405–1411.

Chapter 3

Microbial patterns during *sesotho* fermentation

3.1 Abstract

The growth and succession of microorganisms in fermented foods play an important role as it is responsible for enhancing food properties such as shelf-life, taste, and aroma as well as enhancing the nutritional value. The main aim of this chapter was to quantify and characterize the microbial patterns associated with *sesotho* (an alcoholic indigenous fermented maize and sorghum product) during processing, in order to establish its safety efficacy. The study was carried out in five regions across Lesotho representing the eastern, western, southern, northern and central regions. A total of 25 different fermentation processes were scientifically investigated from the initial stages to the final product, including five locally different processes in each region. Five sampling points during the processing were carefully selected in order to get well-defined representative microbial patterns during the fermentation process. Carbon copy batches were also performed in the laboratory, using the same traditional procedures as the local breweries in Lesotho, to assess and quantify the microbial dynamics during the fermentation process. From all the microbial dynamics assessed in Lesotho and those obtained from batches prepared in the laboratory, the microbial patterns followed similar trends in each region despite variances in brewers and geographical area. The pH was monitored during the progress of all fermentations and a substantial decrease was evident (pH ranging from 5.82 to 3.64) corresponding with an increase in lactic acid bacterial (LAB) numbers ranging from 3.2 - 7.5 log CFU/ml. An increase in yeast numbers from non-detectable - 8.2 log CFU/ml was observed as fermentation progressed. An apparent stabilisation in yeast and LAB numbers towards the end of the fermentation process suggested possible symbioses between the two dominant groups of microorganisms as typical antagonism was initially evident when enteric bacteria declined in numbers from 3.3 log CFU/ml to non-detectable levels in the later stages of the process. This observation indicated that *sesotho* promises a good safety record in terms of the efficacy of regulating the survival of pathogenic and food spoilage bacteria which were mainly enteric bacteria.

Keywords: Fermented product, *Sesotho*, Microbial dynamics

3.2 Introduction

In general, spontaneous fermentation processes are driven by complex microbial ecosystems. They generally start with high microbial diversity which is sourced from human manipulation, utensils and raw materials. As the fermentation progresses, there is an interaction between the microflora and the substrate matrix and this creates a phenomenal dynamic, at both microbial and biochemical levels. This dynamic is characterized by changes in physicochemical conditions such as pH, temperature, sugars and salts concentrations (Bigot *et al.*, 2015; Giraffa, 2004; Steinkraus, 2002). These biochemical changes generate stress factors for microorganisms present. As a consequence of these generated stress factors, there is a decrease in microbial diversity as the fermentation is progressing and the dominant microflora begin to emerge (Hammes *et al.*, 2005; Mugula *et al.*, 2003; Sahlin, 1999; Smid & Lacroix, 2013).

A considerable amount of research has been carried out on the microbiology of spontaneously fermented cereal based food products. LAB and yeasts have been reported to be the dominating microbial groups (Greppi *et al.*, 2013; Katongole, 2008; Kayodé *et al.*, 2011; Omar & Ampe, 2000; De Vuyst & Neysens, 2005; Yi-Seul Kim, Min-Cheol Kim, Soon-Jin Kim, In-Cheol Park, 2011). LAB produce by-products that inhibit other microorganisms, which are generally pathogenic and food spoilers (Alvarez-Sieiro *et al.*, 2016; Angmo *et al.*, 2016; Jeevaratnam *et al.*, 2005; Yang *et al.*, 2012). As for yeasts, studies have shown that they produce ethanol and this has been reported to inhibit other microbial groups which may also be pathogenic and food spoiling (Giraffa, 2004; Nout et al., 1995; Sahlin, 1999). However, because some studies have indicated that the quality of naturally fermented cereals is poor and variable, there is a growing interest to improve this product through the development of starter cultures. Achievement of this goal depends, however, upon a thorough understanding of the microflora of the naturally fermented cereal. Limited research work has been done to identify and characterize the microflora of some of the Lesotho traditional fermented cereals. *Sesotho* is a naturally fermented cereal based alcoholic beverage commonly produced by the rural people all over Lesotho.

At present, limited information on the microbial spectrum associated with *sesotho* and the differences between regions are available other than the work done by Gadaga *et*

al., (2013). The availability of this information is crucial for the development of the product with improved safety and nutritional quality. Thus, the present study was undertaken to investigate the extent to which yeasts and bacteria occur in this traditionally fermented cereal beer and identify the important yeast species with the ultimate objective of characterizing and evaluating their potential use in future small-scale starter culture development.

3.3 Materials and Methods

3.3.1 Sample collection and identification of beer producing households

Sesotho beer samples were collected from five geographically distinct areas in Lesotho namely, Maseru (west), Mafeteng (south), Thaba-Tseka (central), Butha-Buthe (north) and Mokhotlong (east) (geographic details at the appendices). Five brewing households were visited in each region for the documentation of preparation methods as well as sample collection. A brewing household was identified by a white flag located by the beer producing household. Identification of beer producing households was aided by chiefs (village watchmen) as it is the tradition in Lesotho that when there is a study that is to take place in a concerned village, chiefs are notified and asked for permission to carry out the concerned study.

3.3.2 Sampling methodology

The preparation of *sesotho* is divided into two fermentation phases where two sets of starter cultures are added to each of the respective fermentation phase. Due to the complex nature of the beer preparation, five sampling points (stages during fermentation) were carefully chosen in order to get precise representatives of the microbial patterns during the fermentation process. The first **(1)** sample was taken after an hour following the addition of the first starter culture to initiate the first fermentation phase. The second **(2)** sample was taken after the first fermentation phase (approximately after eight hours). The third **(3)** sample was taken an hour following the addition of the second starter to initiate the second and final fermentation phase. The fourth **(4)** sample was taken prior sieving the beer (this was approximately after eight hours following the second fermentation) to separate the sorghum malt from the beer. The fifth **(5)** sample was taken from the finished product approximately after eight hours of maturation. Samples were aseptically collected in one litre sterile bottles and were immediately placed in a mobile fridge set at 4 °C to prevent changes in

microbial populations. Samples were transported to the Department of Biology at the National University of Lesotho (NUL) where microbial counts were carried out.

3.3.3 Laboratory preparation of *sesotho*

Sesotho was also prepared (starter culture was randomly collected from one of the local households in Maseru) in the laboratory at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, following the traditional documented procedures (fig 3.1). Microbial counts performed on laboratory prepared beer were compared to beer produced at village-level households. To standardize the comparisons, the sampling methodology employed was the same as applied for the brews prepared at local households. For the preparation, sorghum flour/malt (500 g) were mixed with maize flour (500 g) and hot boiled water (4 500 ml) was added, while the mixture was stirred to prevent clump formation. Cooled boiled water (3 000 ml) was added to the hot mixture. Following the addition of cooled water, the whole mixture was then cooled to approximately 30 °C, after which the first starter (500 ml) was added and the mixture allowed to ferment at ambient temperature (25 – 30 °C) for 12 hours. Following the initial fermentation step, the mixture was then cooked for 2 - 3 hours followed by cooling to approximately 30 °C. Once cooled, sorghum malt (500 g) and the second starter (500 g) were added to the mixture and allowed to ferment (ambient temperature) for another 12 hours (~10 000 ml preparation recipe). During preparation, samples were aseptically withdrawn at each designated sampling points for pH determination and microbiological analysis.

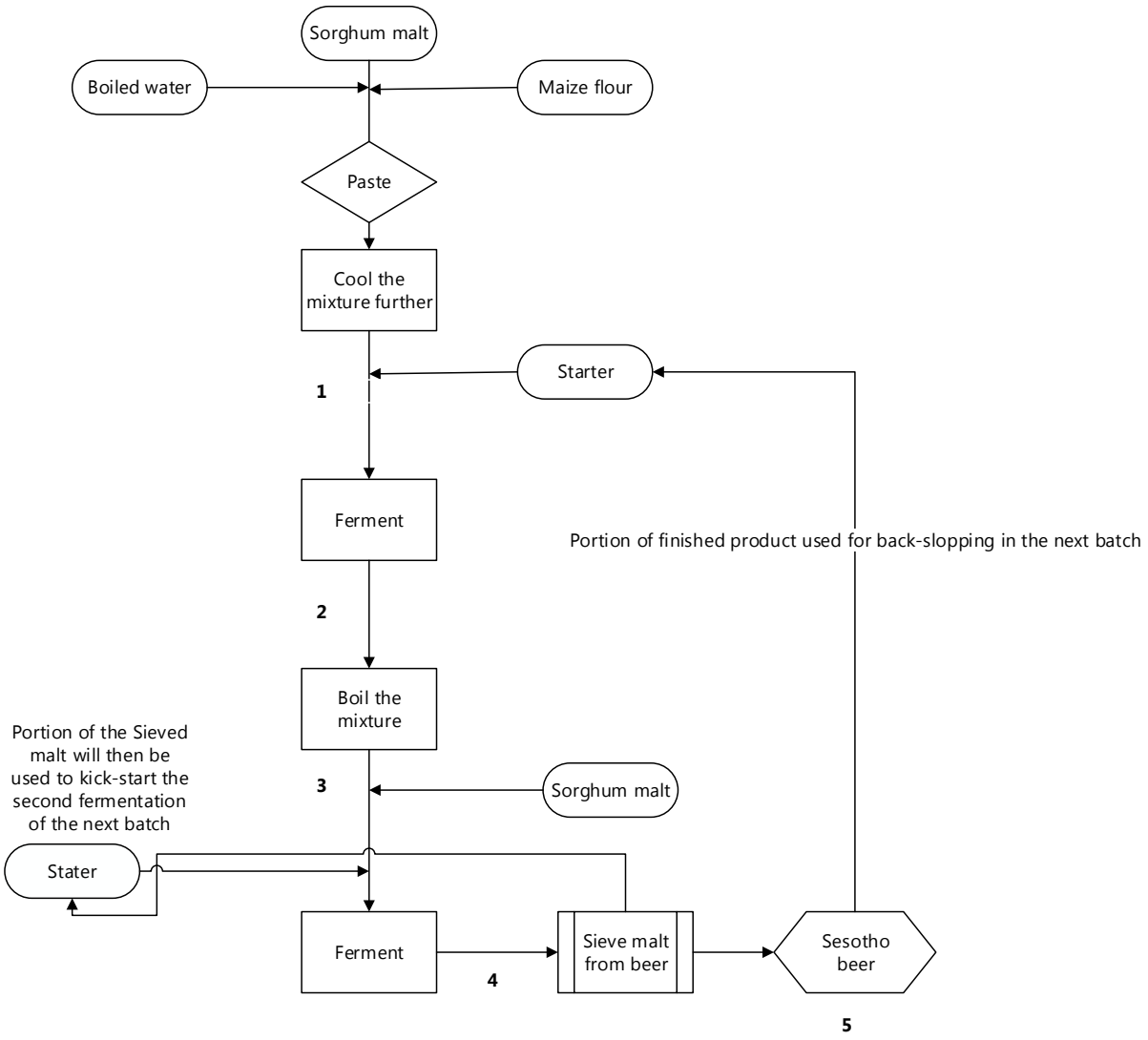

Figure 3.10: Traditional protocol for the preparation of *Sesotho* (sampling points indicated as 1-5).

3.3.3 Enumeration of Lactic acid bacteria, yeasts and coliforms

For standardisation of the microbial enumerations, 10 ml portions of the *sesotho* sample was homogenised with 90 ml of sterile Peptone Physiological Salt solution (Oxoid, Basingstoke, UK). The homogenate was serially diluted and aliquots from appropriate dilutions were used in duplicates on the respective agar media. Numbers of LAB were determined on de Man Rogosa and Sharpe (MRS) and M17 (Biolab, Midrand, RSA) selective media. Appropriate dilutions were spread plated and incubated in ananaerobic flask (Oxoid) in the presence of a gas-generating kit (Anaerobic System BR0038B, Oxoid) at 30 °C for 48 hours. Total aerobic mesophilic counts were obtained by pour plating on PCA and incubating at 30 °C for 48 hours. Coliform counts were obtained by pour plating on Violet Red Bile agar (VRBM) (Oxoid,

Basingstoke, UK) plated and incubated at 37 °C for 24 hours. Yeasts were enumerated by surface plating on Rose Bengal Chloramphenicol Agar (RBCA) (Oxoid, Basingstoke, UK) and aerobically incubated at 25 °C for 3-5 days. Enumerations were performed similarly in both site and laboratory prepared beer samples.

3.3.4 Yeasts identification and characterization

Representatives of yeast colonies were randomly picked from selected RBCA plates and further purified by streaking onto RBCA plates. The pure cultures were then sub-cultured onto Malt Extract (ME) slants and stored at 4 °C. For identification, pure isolates were re-streaked on ME and sent to Inqaba Biotech in Pretoria, South Africa.

Yeast identification was performed through sequence analysis of the D1/D2 domain using primer pair NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5'-GGTCCGTGTTTCAAGACGG) (Kurtzman & Robnett, 1998). Sequencing reactions were performed with the ABI Prism™ Big Dye terminator™ V3.1 cycle sequencing ready reaction kit and data collected on an ABI Prism 377 DNA sequencer (Applied biosystems). Data was analysed using Sequencing analysis V3.3 and sequences assembled using Auto-assembler V1.4.0.

3.3.5 Physico-chemical analysis

For on-site samples in Lesotho, the pH was measured immediately upon the withdrawal of the sample using the HANNA® pocket pH meter (Johannesburg, SA). As for *sesotho* samples collected in the laboratory, the Cyberscan pH 510, (Eutech Instruments) pH meter was used to measure the pH. The pH meter was calibrated using standard buffer solutions (Merck) at pH 4.0 and pH 7.0.

3.3.6 Statistical analysis and graph illustrations

Statistical analysis (data spread) and graphical illustrations depicting the patterns from all 25 breweries across the country were compared using the Box plots (www.r-project.org).

3.4 Results and discussion

3.4.1 Graphical illustrations of the microbial patterns during *sesotho* preparation

All 25 *sesotho* fermentations conducted in Lesotho followed a similar trend of microbial dynamics despite the different geographical regions at which these fermentations were performed. All 25 brewing sessions with five conducted in each region were grouped together in a representative graph based on averages (graphs shown in the appendices). Following the construction of region representing graphs, all the averages from the representing graphs were combined to formulate a graph representing the general pattern or the trend of the microbial dynamics during *sesotho* fermentation (fig 3.2). Graphical illustrations were carried out using the R software (www.r-project.org).

3.4.2 Physico-chemical analysis

General patterns (figs 3.2 and 3.3) illustrate the changes in pH during the fermentation process of *sesotho* preparation. From both graphs, as the fermentation began, there was an observable decrease in pH ranging from 5.82 to 3.64, this was during the first fermentation stage of approximately 12 hours. Following cooking, there was a slight increase in pH from 3.64 to 4.32. This may be due to the addition of water during the cooking process as lactic acid is a weak and unstable acid (Katongole, 2008). After cooking has been completed and the product has cooled down, the second starter culture was added and there was a significant decline in pH to reach a lowest of 3.68. The observed decrease in pH corresponded to the proportional number of LAB counts, indicative of lactic acid production. Similar patterns have been reported during preparation of cereal based fermented products such *boza*, *pozol, bushera* and u*mqombothi* (Botes *et al.*, 2007; Katongole, 2008; Muyanja *et al.*, 2003; Ben Omar & Ampe, 2000).

3.4.3 Microbiological analysis

The predominant microorganisms in naturally fermented cereal beer proved to be aerobic mesophilic bacteria and lactic acid bacteria (LAB), whereas yeasts totally dominated the second phase of fermentation. Figs. 3.2 and 3.3 demonstrate an increase in LAB counts during the first and second stages of the fermenting process.

Similar trends were observed in all samples despite the geographical differences at which samples were collected at. The initial high counts of LAB prior to fermentation are attributed to the biological surface layer of the pot/vessel that is repeatedly used for each batch produced (Katongole, 2008; Kebede & Viljoen, 2007). The slight decrease following cooking, is due to high temperatures that may have killed some of the LABs. This trend was consistent in all samples at different breweries. The increase in LAB counts following the 2nd fermentation phase is attributed to the addition of the second starter predominated by LAB numbers as this starter is derived from back-slopping (fig. 3.1).

Despite low yeast numbers during the first fermentation phase, yeasts developed rapidly during the second phase to reach counts as high as 8 log units. This observation suggested that yeasts play an important role during the *sesotho* fermentation and may add to final taste and flavour other than only alcohol production. During the initial stages (first fermentation phase), polymers (starches) are broken down to monomers (simple sugars) and then these sugars serve as fermentation substrates for yeasts to yield alcohol. Yeasts are also known to produce flavour compounds such as organic acids, aldehydes and esters. These compounds may be important in the final characteristics of the product. In addition, yeasts are known to stimulate lactic acid production by LAB (Kayodé *et al.*, 2011; Oro, 2013).

Results also showed (figs. 3.3 and 3.2) a substantial decrease in coliform numbers from an initial average count of 3.2 log units to non-detectable levels after 12 hours followed by an increase directly after cooking/boiling and the addition of the second starter culture and sorghum malt. This increase in numbers was possibly due to the fact that brewers at this particular stage manually mix the starter culture and the sorghum malt with the brew. This trend was however also observed in all samples, including the laboratory samples, indicating that it was more likely that the coliforms originated from the starter culture. These starters are usually only kept in a pot covered with a piece of cloth after obtaining it from previous fermentations. The un-hygienic storage of back-slope starters remains a serious risk factor and a crucial stage for hazard safety analysis (fig. 3.1). However, again the number of coliforms declined to non-detectable levels towards the end of fermentation. The overall decrease of enteric bacteria during the fermentation coincided with the lower pH levels mainly caused by the lactic acid produced by LAB. In addition to the acidic environment created by LAB,

there are also compounds such as bacteriocins and ethanol that are inhibitory to the enteric bacteria (Caplice & Fitzgerald, 1999). The presence of coliforms especially above log 2 CFU/ml calls for concern as this indicates contamination by spoilage and disease-causing enteric bacteria (Katongole, 2008). It is interesting to note that both laboratory prepared and site prepared *sesotho* fermentations followed the same trend of dynamics (figs. 3.2 and 3.3).

3.4.4 Microbial interactions

Figures 3.3 and 3.2 depict LAB dominating the initial stages whereas yeasts only emerged after the initial fermentation phase (12 hours) and substantially towards the end of the second fermentation phase. The rapid increase of yeast numbers during the second stage of fermentation may be attributed to the production of alcohol at this time and thereby inhibiting or retarding the growth and proliferation of other groups of microorganisms. The stabilization in LAB and yeast numbers at the end of fermentation suggests the end of typical antagonism and possibly a symbiotic relationship between the organisms. This relationship has been suggested by several researchers (Al-Otaibi, 2012; Katongole, 2008; Kingamkono *et al.*, 1998; Omar & Ampe, 2000; Oro, 2013). Most notably, yeast species encountered such as *Saccharomyces cerevisiae, Issatchenkia* and *Candida krusei* were reported to be the dominant microorganisms during the fermentations of several cereal-based products such as *pito, bushera, boza, umqombothi* and *tchoukoutu* (Giraffa, 2004; Gotcheva *et al.*, 2000; Katongole, 2008; Kayodé *et al.*, 2011; Muyanja *et al.*, 2003). *Issatchenkia* species were less encountered during previous studies. *Saccharomyces* has been reported to stimulate the growth of some LAB as well as providing essential metabolites such as pyruvic acid, amino acids and vitamins. Inhibition of enteric bacteria could be attributed by the acidic and alcoholic environment created by both LAB and yeasts (Caplice & Fitzgerald, 1999; Nout, 1994). This could also be attributed to the production of bacteriocins produced by LAB during the fermentation process (Achi & Ukwuru, 2015; Caplice & Fitzgerald, 1999; Chen & Hoover, 2003; Deegan *et al.*, 2006; Holzapfel *et al.*, 1995). The high numbers of coliforms in the product, however reinforces the need for fast acid producing starter cultures which can retard the proliferation of the coliforms before they reach high numbers. The high coliform numbers may be

detrimental to the product causing off-flavours, shortening the shelf-life and adding to the quality risk factor.

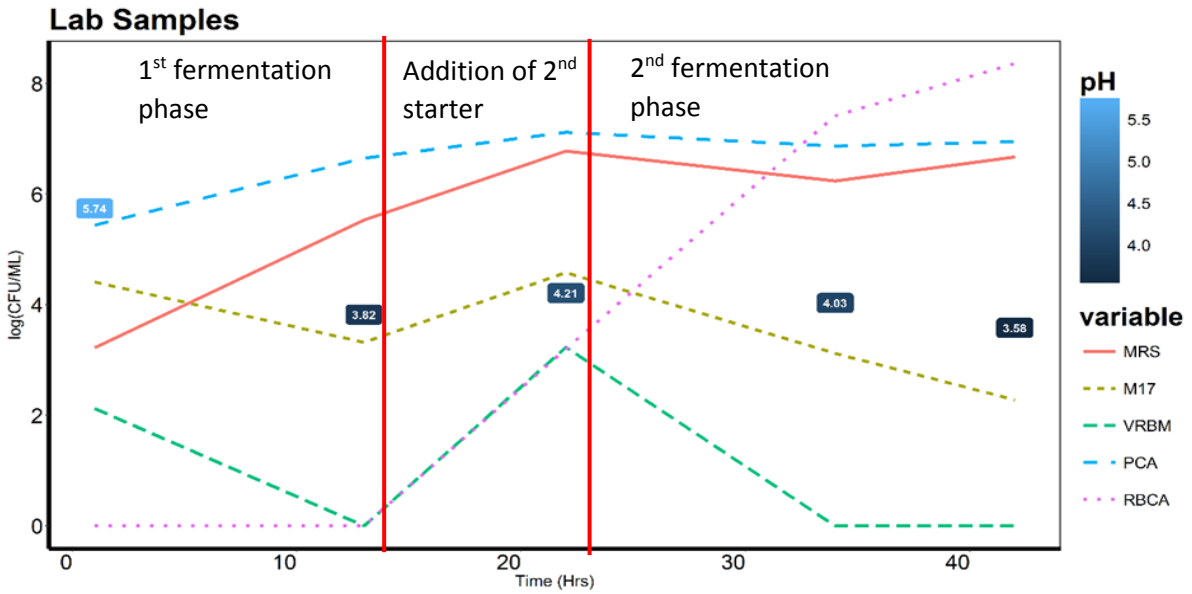

Figure 3.2: Microbial patterns during the *sesotho* fermentation performed in the laboratory.

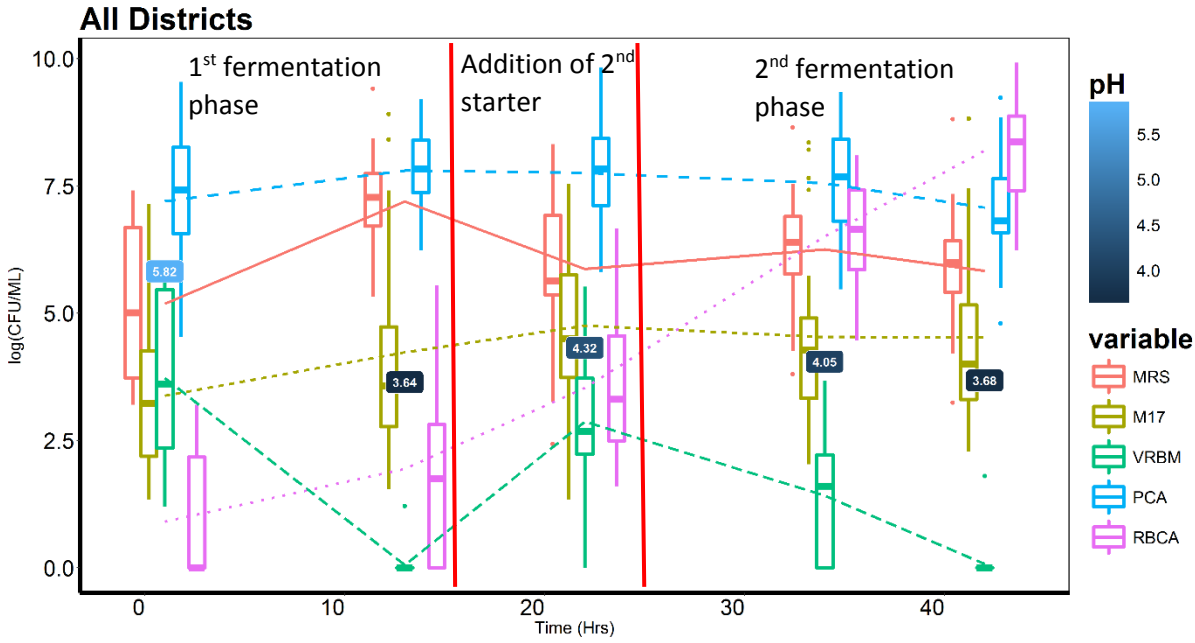

Figure 3.3: Microbial patterns of *sesotho* fermentation in all districts surveyed.***Box plots indicate the range of counts**

3.4.4 Dominant LAB

Dynamics of LAB were followed using two types of selective media, MRS and M17. The MRS media specifically selects for lactobacilli whereas M17 specifically selects for streptococci. It is interesting to note that despite different geographical regions where *sesotho* was brewed, the general dynamics were dominated by bacteria belonging to the group lactobacilli.

3.4.5 Yeast identification

Taxonomic trees were then constructed to characterise the yeasts obtained from the beer samples. Despite the geographical differences where the beers were brewed, from all the samples, the predominant yeasts were identified to belong to the genera of *Saccharomyces, Issatchenkia* and *Pichia* Figs. 3.4 and 3.5).

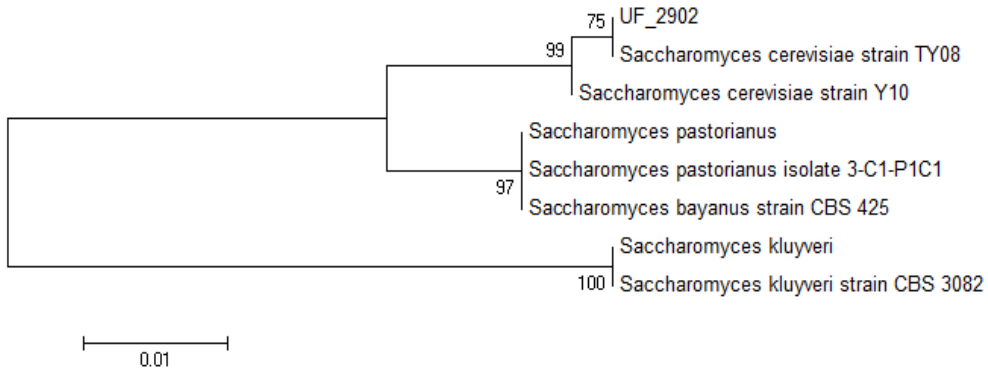

Figure 3.4: Taxonomic tree of yeasts isolated from *sesotho*

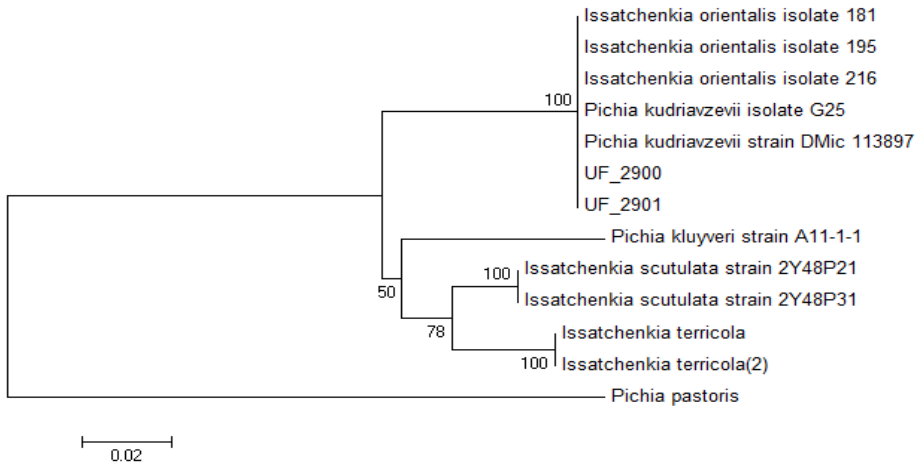

Figure 3.5: Extended taxonomic tree of yeasts isolated from *sesotho.*

3.5 Conclusions

The microbiota of naturally fermented cereal beer during the initial stages was predominated by aerobic mesophilic bacteria and lactic acid bacteria. However, considerable growth of yeasts was observed during the second stage of the fermentation process becoming predominant. The total exchange in predominance between yeasts and LAB during the *sesotho* fermentation was unexpected as some studies reported an almost co-dominant dynamics of LAB and yeasts during fermentation. However, there was an observable stabilization in LAB (predominantly lactobacilli) and yeasts population numbers at the later stages of the fermentation process was evident suggesting a possible symbiotic association between these groups. From the study it has also been established that irrespective of the geographic region from which the beer was brewed, the changes in the microbial patterns remained similar. Dominating yeasts in the s*esotho* preparation were representatives mainly from the genera *Pichia, Issatchenkia* and *Saccharomyces*.

The study also clearly demonstrated the inhibition of enteric bacteria by the changes present during the fermentation process suggesting a good fermentation efficacy as well as a good safety record for *sesotho* despite the lack of proper hygienic circumstances of different producers. Nonetheless, some sampling points appeared to harbour high loads of coliforms. The high numbers of coliforms in the product, however reinforces the need for fast acid producing starter cultures which can retard the proliferation of the coliforms before they reach high numbers. The high coliform numbers may be detrimental to the product causing off-flavours, shortening of the shelf-life and adding to the quality risk factor. The growth of lactic acid bacteria initiating lower pH levels correlated to the sufficient inhibition of coliform bacteria and therefore may be a dependable means of controlling the growth of coliform bacteria. Alcohol production however, may have also aided to the inhibition of coliforms.

More in-depth studies on the microbial ecology of *sesotho* are essential as up to now little is known about the microbial interplay during the fermentation process. Due to inherent bias of culture-dependent techniques. Therefore it is important to utilise modern molecular techniques to further confirm the precise microbial

representatives of the ecosystem (Ben Omar & Ampe, 2000). The application of unbiased, standardised techniques to assess *sesotho* from different geographical regions will aid in reaching a consensus regarding the definition of *sesotho* microbial ecosystem (Marsh *et al.*, 2014; Morozova & Marra, 2008; Shendure & Ji, 2008). This will serve as a crucial stepping stone towards the development of the starter culture as it is one of the major steps towards improving the safety, quality, and security of traditional production of *sesotho*.

3.6 References

Achi, O. K. & Ukwuru, M. (2015). Cereal-Based Fermented Foods of Africa as Functional Foods. *Int J Microbiol Appl* **2**, 71–83.

Al-Otaibi, M. M. (2012). Isolation and identification of lactic acid bacteria and yeasts from Sameel milk: A Saudi traditional fermented milk. *Int J Dairy Sci* **7**, 73–83.

Alvarez-Sieiro, P., Montalbán-López, M., Mu, D. & Kuipers, O. P. (2016). Bacteriocins of lactic acid bacteria: extending the family. *Appl Microbiol Biotechnol* **100**, 2939–2951.

Angmo, K., Kumari, A., Savitri & Bhalla, T. C. (2016). Probiotic characterization of lactic acid bacteria isolated from fermented foods and beverage of Ladakh. *LWT - Food Sci Technol* **66**, 428–435.

Bigot, C., Meile, J.-C., Remize, F. & Strub, C. (2015). Applications of Metagenomics to Fermented Foods. In *Fermented Foods, Part I (Biochemistry Biotechnol*, pp. 333–346. Edited by C. R. Didier, Montet; Ramesh. CRC Press 2015.

Botes, A., Todorov, S. D., von Mollendorff, J. W., Botha, A. & Dicks, L. M. T. (2007). Identification of lactic acid bacteria and yeast from boza. *Process Biochem* **42**, 267–270.

Caplice, E. & Fitzgerald, G. F. (1999). Food fermentations: Role of microorganisms in food production and preservation. *Int J Food Microbiol* **50**, 131–149.

Chen, H. & Hoover, D. G. (2003). Bacteriocins and their Food Applications. *Compr Rev Food Sci food Saf* **2**, 82–100.

Deegan, L. H., Cotter, P. D., Hill, C. & Ross, P. (2006). Bacteriocins: Biological tools for bio-preservation and shelf-life extension. *Int Dairy J* **16**, 1058–1071.

Gadaga, T. H., Lehohla, M. & Ntuli, V. (2013). Traditional Fermented Foods of Lesotho. *J Microbiol Biotechnol Food Sci* **2**, 2387–2391.

Giraffa, G. (2004). Studying the dynamics of microbial populations during food fermentation. *FEMS Microbiol Rev* **28**, 251–260.

Gotcheva, V., Pandiella, S. S., Angelov, A., Roshkova, Z. G. & Webb, C. (2000). Microflora identification of the Bulgarian cereal-based fermented beverage boza. *Process Biochem* **36**, 127–130.

Greppi, A., Rantsiou, K., Padonou, W., Hounhouigan, J., Jespersen, L., Jakobsen, M. & Cocolin, L. (2013). Determination of yeast diversity in ogi, mawe, and tchoukoutou by using culture-dependent and -independent methods. *Int J Food Microbiol* **165**, 200–207.

Hammes, W. P., Brandt, M. J., Francis, K. L., Rosenheim, J., Seitter, M. F. H. & Vogelmann, S. A. (2005). Microbial ecology of cereal fermentations. *Trends Food Sci Technol* **16**, 4–11.

Holzapfel, W. H., Geisen, R. & Schillinger, U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *Int J Food Microbiol* **24**, 343–362.

Jeevaratnam, K., Jamuna, M. & Bawa, A. S. (2005). Biological preservation of foods - Bacteriocins of lactic acid bacteria. *Indian J Biotechnol* **4**, 446–454.

Katongole, J. (2008). *The Microbial Succession in Indigenous Fermented Maize Products*. University of the Free State.

Kayodé, a P. P., Linnemann, a R. & Kotchoni, S. O. (2011). Diversity of yeasts involved in the fermentation of tchoukoutou , an opaque sorghum beer from Benin. *J Microbiol* **5**, 2737–2742.

Kebede, A. & Viljoen, B. C. (2007). The effect of container type on the growth of yeast and lactic acid bacteria during production of Sethemi , South African spontaneously fermented milk. *Food Res Int* **40**, 33–38.

Kingamkono, R. R., Sjögren, E. & Svanberg, U. (1998). Inhibition of enterotoxin production by, and growth of enteropathogens in a lactic acid-fermenting cereal gruel. *World J Microbiol Biotechnol* **14**, 661–667.

Kurtzman, C. P. & Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie Van Leeuwenhoek* **98**, 331–332.

Marsh, A. J., Hill, C., Ross, R. P. & Cotter, P. D. (2014). Fermented beverages with potential : Past and future perspectives. *Trends Food Sci Technol* **38**, 113–124.

Morozova, O. & Marra, M. A. (2008). Genomics Applications of next-generation sequencing technologies in functional genomics. *Genomics* **92**, 255–264.

Mugula, J. K., Nnko, S. a M., Narvhus, J. a. & Sørhaug, T. (2003). Microbiological and fermentation characteristics of togwa, a Tanzanian fermented food. *Int J Food Microbiol* **80**, 187–199.

Muyanja, C. M. B. K., Kikafunda, J. K., Narvhus, J. A., Helgetun, K. & Langsrud, T. (2003). Production methods and composition of Bushera: a Ugandan traditional fermented cereal beverage. *African J Food, Agric Nutr Dev* **3**, 10–19.

Nout, M. J. R., Kok, B., Vela, E., Nche, P. F. & Rombouts, F. M. (1995). Acceleration of the fermentation of kenkey, an indigenous fermented maize food of Ghana. *Food Res Int* **28**, 599–604.

Nout, M. (1994). Fermentation and food safety. *Food Res Int* **27**, 291–298.

Omar, N. & Ampe, F. (2000). Microbial Community Dynamics during Production of the Mexican Fermented Maize Dough Pozol. *Appl Environ Microbiol* **66**, 3664–3673.

Oro, L. (2013). *Role of yeast bioactive compounds in food and fermented beverages*. Polytechnic University of Marche.

Sahlin, P. (1999). *Fermentation as a Method of Food Processing production of organic acids, pH-development and microbial growth in fermenting cereals*. Lund University.

Shendure, J. & Ji, H. (2008). Next-generation DNA sequencing. *Nat Biotechnol* **26**, 1135–1145.

Smid, E. J. & Lacroix, C. (2013). Microbe-microbe interactions in mixed culture food fermentations. *Curr Opin Biotechnol* **24**, 148–154.

Steinkraus, H. K. (2002). Fermentations in world food processing. *Compr Rev food Sci food Saf* **1**, 23–32.

De Vuyst, L. & Neysens, P. (2005). The sourdough microflora: Biodiversity and metabolic interactions. *Trends Food Sci Technol* **16**, 43–56.

Yang, E., Fan, L., Jiang, Y., Doucette, C. & Fillmore, S. (2012). Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. *AMB Express* **2**, 48.

Yi-Seul Kim, Min-Cheol Kim, Soon-Jin Kim, In-Cheol Park, J.-O. and H.-Y. W. (2011). Analyses of bacterial communities in Meju, a Korean traditional fermented soybean bricks, by cultivation-based and pyrosequencing methods. *J Microbiol* **49**, 340–348.

Chapter 4

Bacterial diversity and distribution during the *sesotho* fermentation process

4.1 Abstract

Sesotho is an indigenous cereal based fermented beer produced in Lesotho, its microbial ecosystem is derived from raw ingredients, utensils and indigenous starter cultures. Currently there is no knowledge about the bacterial ecosystem of *sesotho*. In this study, 25 beer samples from five geographically distinct locations across Lesotho were collected periodically as the fermentations progressed. The Next Generation Sequencing (NGS) of hypervariable V1/V3 region of the 16S ribosomal RNA (rRNA) was used for the bacterial community profiling. Results showed that the bacterial diversities followed similar patterns in all the fermentation processes despite different geographical locations and different brewers. The study demonstrated that various genera of LAB such as *Enterococcus, Pediococcus*, *Lactobaccilus*, *Leuconostoc* and W*iesella* were dominant during the fermentation processes. The results also demonstrated the potential of the safety efficacy of the process as the microbial groups with pathogenic and spoilage history declined as the fermentation was progressing. However, the results showed that as much as there is a decline in Gammaproteobacteria, safety does not entirely lie on the fermentation process itself but also on the raw ingredients, utensils and the brewer. Results demonstrated that the brewer influenced about 28% of the bacterial diversity. This study provides a comprehensive understanding of the bacterial diversity of s*esotho.*

Keywords: Bacterial diversity, Sesotho, Next generation sequencing, Safety

4.2 Introduction

Indigenous fermented foods represent a large portion of human daily diets and are consumed world-wide (Achi, 2005; Marshall & Mejia-Lorio, 2012). Most of the fermentation processes are spontaneous and driven by complex microbial communities (i.e. bacteria, yeasts and moulds) sourced from raw ingredients, utensils, starter culture inoculation which influence the organoleptic quality, nutrient availability as well as the safety of the fermented product (Chuah *et al.*, 2016; Hwanhlem *et al.*, 2014; Martinez *et al.*, 2015). Since lactic acid bacteria (LAB) play a dominant and prominent role in cereal based fermentations. The scientific and industrial interests within the food biotechnology fraternity have increased towards investigations of LAB ecological roles and functionalities (Park *et al.*, 2012).

The complex microbial ecosystem behind the fermentation process can constitute of LAB that are not cultivable and in addition to this, similar physiological properties that contribute to similar food characteristics may potentially result from phylogenetically distant bacterial species (Bigot *et al.*, 2015). As a consequence, conventional taxonomic methods proofed not to be the best tools to study the bacterial diversity of food fermentations as they can give an underestimated, filtered view and potentially misleading picture about the bacterial composition of fermented products in question (Cocolin *et al.*, 2013).

Spontaneous food fermentation normally starts with a high microbial diversity derived from microbial loads on raw materials, utensils and the processor's hands (Achi & Ukwuru, 2015; Minervini *et al.*, 2014). Fermentation processes are governed by these spontaneous species present and further characterised by the biochemical modification of food composition. For instance, LAB fermentation produces lactic acid and in addition acetic acid, carbon dioxide and ethanol depending on the biochemical and environmental circumstances (Steinkraus, 2002). Consequently, throughout the fermentation process there is an interaction between the microflora and the substrate matrix and this creates interesting dynamics both at biochemical and microbial levels. Furthermore, these dynamics change the physico-chemical conditions such as temperature, water activity, pH, sugar and salt concentrations (Bigot *et al.*, 2015). All these variable biochemical changes generate stress factors that eventually alter the microbial communities. For instance, alcohol produced causes some impairments of

microbial cell membrane properties. An increase of organic acid levels creates a dissipation of the proton gradient throughout the cellular membrane (Caplice & Fitzgerald, 1999). Production of other antibacterial agents such as bacteriocins (produced by LAB) will inhibit other bacteria who are sometimes pathogenic or food spoiling and thus reducing the microbial diversity (Ahmad *et al.*, 2016; Vera-Pingitore *et al.*, 2016). Due to the production of organic acids, the pH will decrease and retard or kill other microorganisms that are acid intolerant (Masha *et al.*, 1998). As a result of a fermentation progression, microbial diversity decreases over time and the surviving (dominant) microbiota appears (Assohoun-Djeni *et al.*, 2016; Escobar-Zepeda *et al.*, 2016; Muyanja *et al.*, 2003; Ruiz Rodríguez *et al.*, 2016).

Over the last ten years molecular techniques have been emerging as accurate and reliable identification tools and are being used in combination with culture-dependent techniques to complement the microbial description of environmental samples (Cason, 2015; Ye *et al.*, 2016). Metagenomics is a Next Generation Sequencing (NGS) tool and is described as the application of modern genomic tools to study the microbial communities directly from their natural environments without the need for isolation and laboratory cultivation of individual species (Kergourlay *et al.*, 2015; Morozova & Marra, 2008). Contrary to conventional culture-dependent methods, metagenomics approaches provide a more comprehensive, quicker way to describe the microbial diversity *in situ*. Metagenomics techniques rely on DNA/RNA (nucleic acids) analyses and are associated with massive sequencing tools and bioinformatics (Mozzi *et al.*, 2013; Shendure & Ji, 2008; Yi-Seul Kim, *et al*., 2011). Application approaches of metagenomics by 16S ribosomal RNA (rRNA) gene sequence based sequencing allows a comprehensive and high throughput analysis of microbial diversity within an environment and this method has recently been used for describing the microbial profiles of various traditional fermented foods (Botta and Cocolin, 2012).

Currently there is no information on the microbial diversity of *sesotho*. The main objective of this study is to construct a thorough insight of the bacterial diversity and distribution over time during *sesotho* fermentation. We characterised the bacterial diversity and community composition of *sesotho* beer brewed at five geographically distinct areas in Lesotho using the Illumina MiSeq platform to sequence the ubiquitously hypervariable bacterial V3/4 region of 16S rRNA. This was pursued to obtain a comprehensive bacterial definition of s*esotho*.

4.3 Materials and methods

4.3.1 Collection of brewing samples from rural sites

Beer samples were collected from five geographically distinct areas in Lesotho namely, Maseru (west), Mafeteng (south), Thaba-Tseka (central), Butha-Buthe (north) and Mokhotlong (east) (fig. 4.1). Five breweries were visited, from each region, for the documentation of preparation methods as well as sample collection. A brewing household was identified by a white flag located by the beer producing household (brewery) (fig. 4.2). Identification of beer producing households was aided by chiefs (village watchmen) as it is the tradition in Lesotho that when there is a study that is to take place in a concerned village, chiefs are notified and asked for permission to carry out the concerned study.

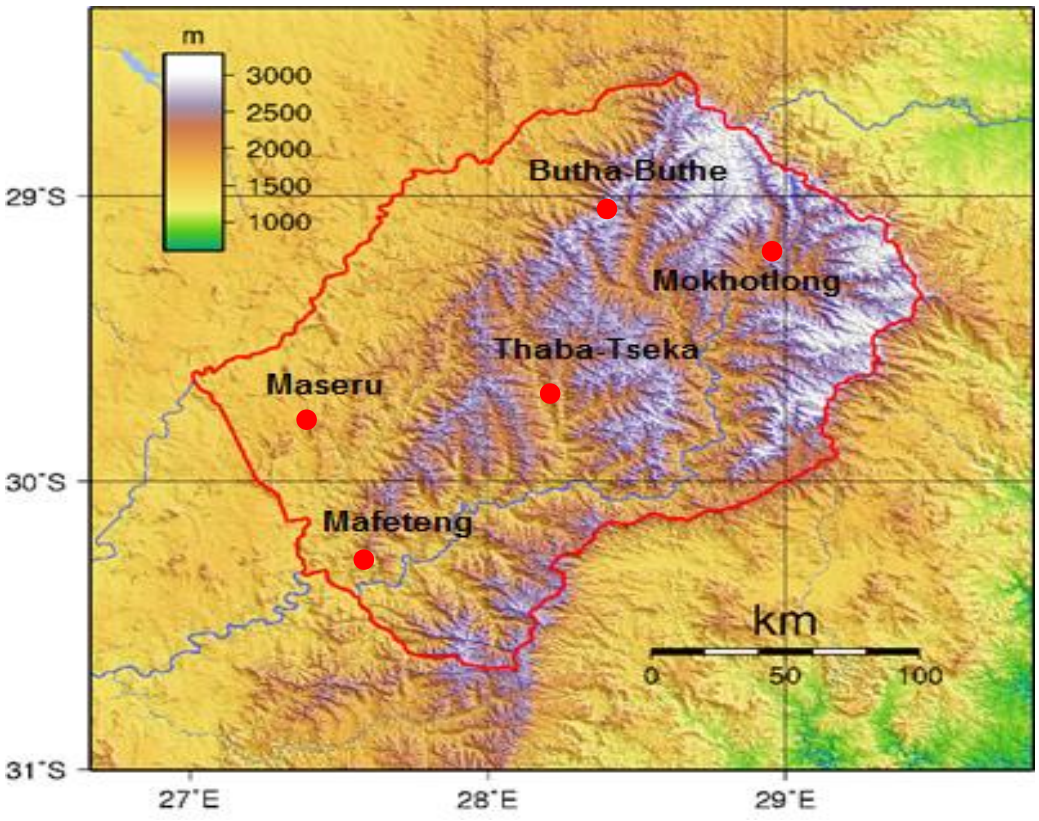

Figure 4.1: A topographical map of Lesotho indicating sampling sites.

Figure 4.2: Local *sesotho* brewing households with white flags.

4.3.2 Fermentation monitoring and sampling

The bacteria shifts during the fermentation of *sesotho* was elucidated by periodically obtaining samples at 5 points during the fermentation process as shown in Figure 4.3. The first sample **(1)** was taken after an hour following the addition of the first starter culture to initiate the first fermentation phase. The second sample **(2)** was taken after the first fermentation phase (approximately after 12 hours). The third sample **(3)** was taken an hour following the addition of the second starter to initiate the second and final fermentation phase. The fourth sample **(4**) was taken prior to sieving the beer (separating the sorghum malt from the beer). This was approximately after eight hours following the second fermentation. The fifth sample **(5)** was taken from the then finished product approximately at 8 hours into maturation. There were therefore 5 samples from each district, Maseru samples being MSU1-5, Butha Buthe (BU1-5), Mokhotlong (MK1-5), Mafeteng (MFT1-5), Thaba-Tseka (HN1-5) and Mafeteng (MFT1-5). Samples were aseptically collected in one litre (1 L) sterile bottles and were immediately placed in a mobile freezer set at -21°C to prevent any microbial changes. Samples were transported to the Department of Microbial, Biochemical and Food Biotechnology at the University of the Free State (UFS) in South Africa, where they were thawed and prepared for NGS and chemical analyses.

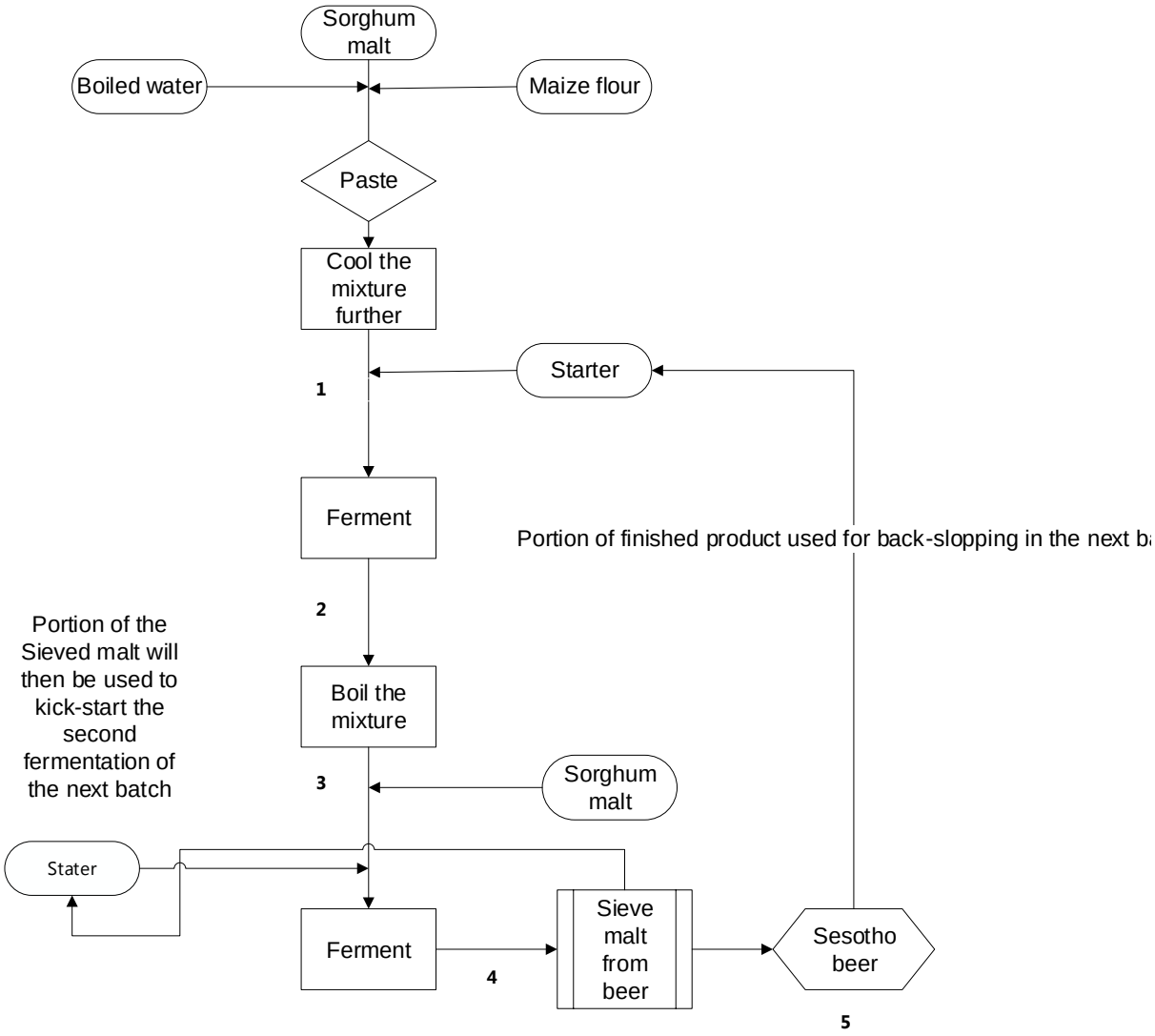

Figure 4.3: A flow diagram of *sesotho* preparation and sampling points (1-5).

Following thawing of frozen samples, a portion of each sample (5 ml) was centrifuged using Beckman centrifuge J2-21 (Beckman, USA) (2500 rcf for 1 minute) and the supernatant was discarded. The pellet was washed twice with buffer (100 mM Tris-EDTA, pH 8) and the supernatant was discarded following centrifugation (2500 rcf for 1 minute). Following washing with buffer, pellets were re-suspended in buffer (100 mM TE, pH 8). Obtained pellets were screened for the presence of bacteria by gram straining (fig. 4.4) (Thairu *et al.*, 2014). Prior to shipment, pellets were frozen at -80°C and transported on dry ice to the Centre of Proteomics and Genomic Research (CPGR) in Cape Town, South Africa, for genomic DNA extraction, library preparation and sequencing.

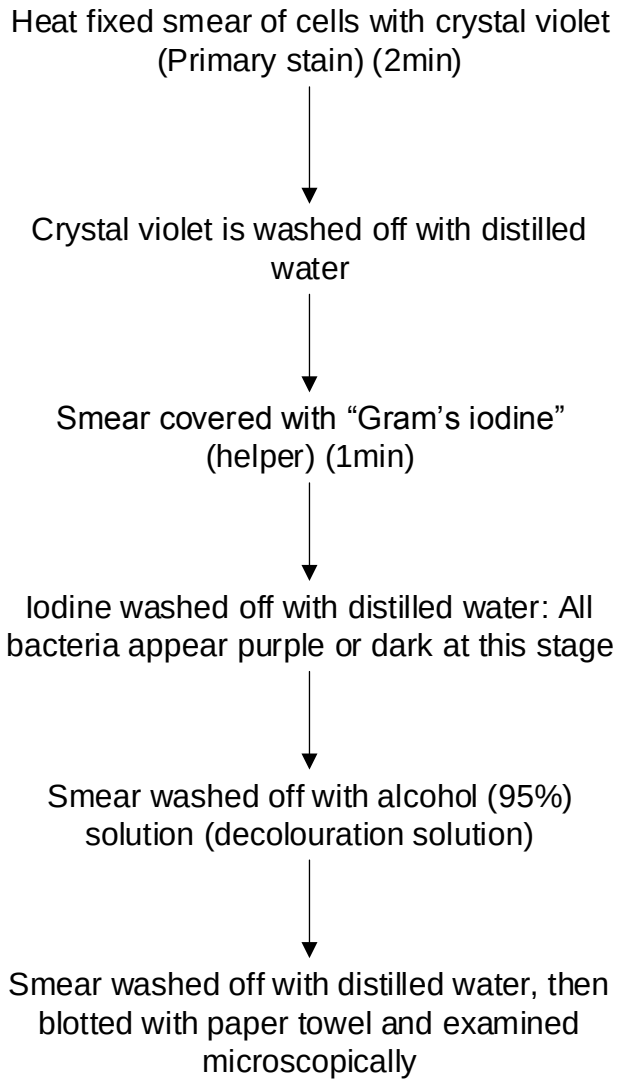

Figure 4.4: Gram stain procedure (Thairu *et al.*, 2014).

4.3.4 16S rRNA metagenomics sequencing data analysis

The sequencing library was constructed at the Centre for Proteomic and Genomic Research, Cape Town, South Africa. Briefly, genomic DNA was extracted and the sequencing library was prepared by amplifying a ~460 bp region located in the hypervariable V3/4 region of the 16S rRNA gene using region of interest-specific primers (Klindworth *et al.*, 2013) with overhang Illumina adapter overhang nucleotide sequences. The 16S V3/4 amplicons were purified using the Agencourt AMPure XP bead clean up kit (Beckman Coulter Genomics, Danvers, MA, USA), followed by a second amplification to attach dual indices and Illumina sequencing adapters using the Nextera XT Index kit (Illumina, San Diego, CA, USA). Final purification using the AMPure XP bead clean up kit (Beckman Coulter Genomics) was done, followed by

library quantification, normalization, pooling and denaturing before being subjected to 2 x 301 cycle sequencing on the Illumina MiSeq using the MiSeq v3 reagent kit (Illumina).

The obtained 16S rRNA sequence data was analysed using QIIME as described by (Caporaso *et al.*, 2011) with alterations. Briefly, before running the QIIME pipeline, the quality of the sequencing was assessed and quality control performed using PrinSeq-lite v0.20.4 (Schmieder & Edwards, 2011). All data sets were pre-processed and trimmed to obtain an average quality score of ≥20 using a 5 nt window with a 3 nt step. All sequences shorter than 200 bp were filtered out and paired end reads merged using PEAR 0.9.6 (Zhang *et al.*, 2014). The demultiplex and quality filtering script in QIIME was run without any additional inputs to obtain a FASTA output file that could be analysed in the QIIME pipeline. Chimeric sequences were identified, using usearch 6.1.544 (Edgar, 2010) as the chimera detection method against the RDP "Gold" database (Edgar, 2010), and filtered out of the quality trimmed reads by using identify_chimeric_seqs.py and filter_fasta.py commands, respectively, in QIIME. Operational taxonomic unit (OTU) picking was carried out and taxonomy assigned to representative OTUs using the pick_open_reference_otus.py script, at 97% sequence identity against the SILVA 119 database (Quast *et al.*, 2013).

4.3.5 Physico-chemical analysis

The pH of each sample was measured instantly on site following withdrawal using a HANNA® pocket pH meter (Johannesburg, SA). Following the arrival of frozen samples, they were thawed and a portion of each sample (5 ml) was centrifuged at 10 000 rcf for 5 minutes using a Beckman centrifuge J2-21 centrifuge (Beckman, USA). The supernatant was then collected for acid analyses by High Performance Liquid Chromatography (HPLC). Acetate, Lactate, glucose and glycerol were analyzed on a Finnigan Surveyor plus HPLC fitted with a Biorad Aminex HPX 87H ion exchange column, 300mm x 10mm. The Eluant was 5 mM, sulphuric acid at a flow rate of 0.6 ml/min. Acids were detected with a PDA detector at 202 nm and glycerol and glucose with a refractive index detector connected in series. Analytes were quantified by external standard (Katongole, 2008).

Gas Chromatography (GC) analysis of ethanol content was carried out with a Shimadzu 2010 instrument fitted with a CPWax 52CB column of dimensions 30 m (L)

x 0.32 mm (ID) x 0.25 µm (Film thickness). The Carrier gas was hydrogen at a linear velocity of 60cm/s. The flame ionization detector temperature was 260 °C and that of the injection port was 150 °C. Injection volume was 1 µL with a split of 1:10. The initial column temperature was 40 °C held for 3 minutes and then ramped to 256 °C at 25 °C/min and held for 1 minute. Shimadzu GC Solution software was used for instrument control, data collection and analysis (Katongole, 2008).

4.3.6 Statistical analysis

Microbial alpha diversities were analyzed in QIIME with alpha_rarefaction.py using the Shannon diversity metric. Beta-diversities were analyzed using Principal Coordinates Analysis (PCoA) plots in R (www.r-project.org) using “plot ordination” in the “Phyloseq” package using Bray-curtis distance (McMurdie & Holmes, 2013). Prior to beta-diversity analysis the OTU-table was normalized using normalize_table.py in QIIME with the CSS normalization option (Paulson *et al.*, 2013).

4.4 Results and discussion

4.4.1 Gram stain results

The gram stain technique was employed to confirm if the washed pellets were indeed bacteria before they were sent to CPGR for sequencing. The gram stain results revealed a mixture of bacteria with diverse gram stain properties and morphologies as shown in Figure 4.5.

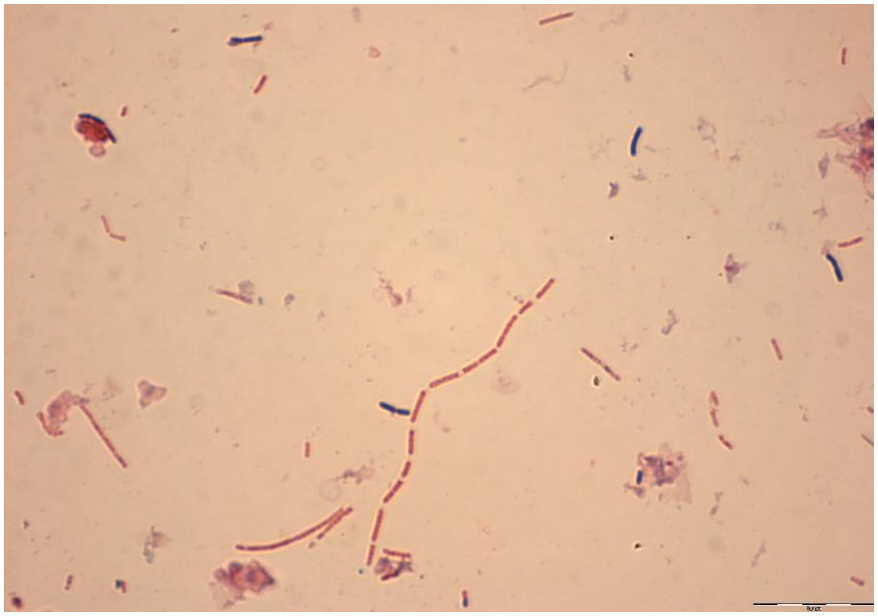

Figure 4.5: Gram stain of screened pellets.

4.4.2 Sequence quality assessment and data filtering

The paired-end library (2x301 bp) resulted in a total average of 178975 good quality sequences obtained across all samples, the average sequence length in all samples was 200-301 bp. The quality score of samples is indicated in figure 4.6.

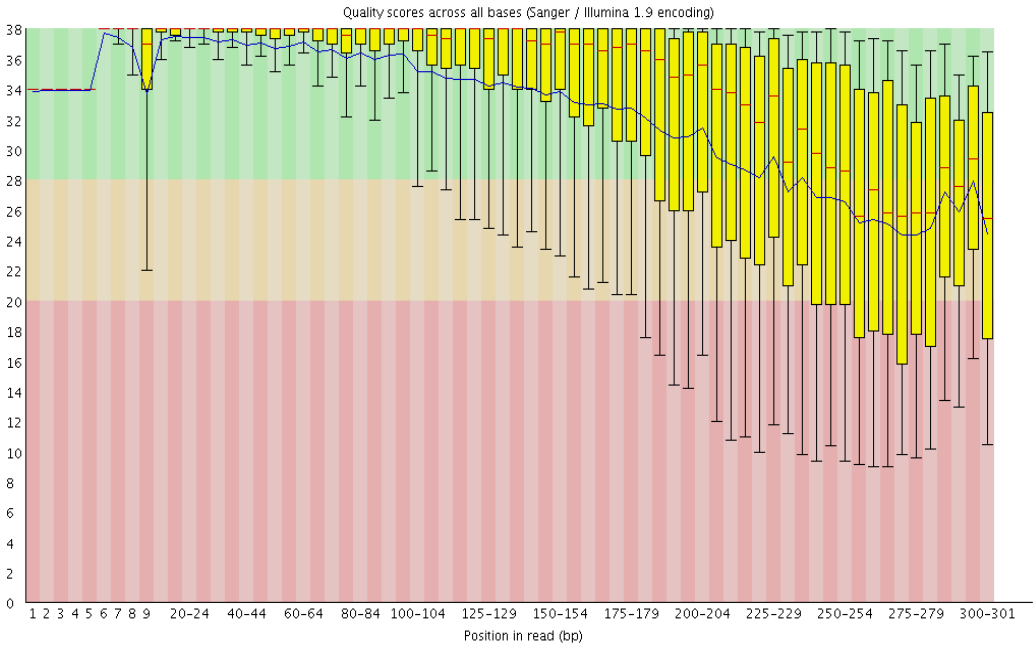

Figure 4.6: Illustration of the sequence quality score (BU1 used as an example).

4.4.3 Bacterial diversity and abundance

The *de facto* standard approach of 16S data analysis starts by grouping reads of similar sequences into OTUs. Generally, the assumption is that sequences assigned to the particular OTU are phylogenetically related to each other and are thus likely to branch from similar ecological sub-populations (Tikhonov *et al.*, 2013). In this study, the OTUs were assigned on the 97% similarity level as this is the standard rule for 16S data analysis. A 97% identity threshold of the 16S gene sequence variants clusters OTUs at genus level. The rarefaction plots indicated that the sampling depth and sequence coverage were good for all the samples across all five brews (fig. 4.7) Samples will be referred to as shown in Figure 4.8.

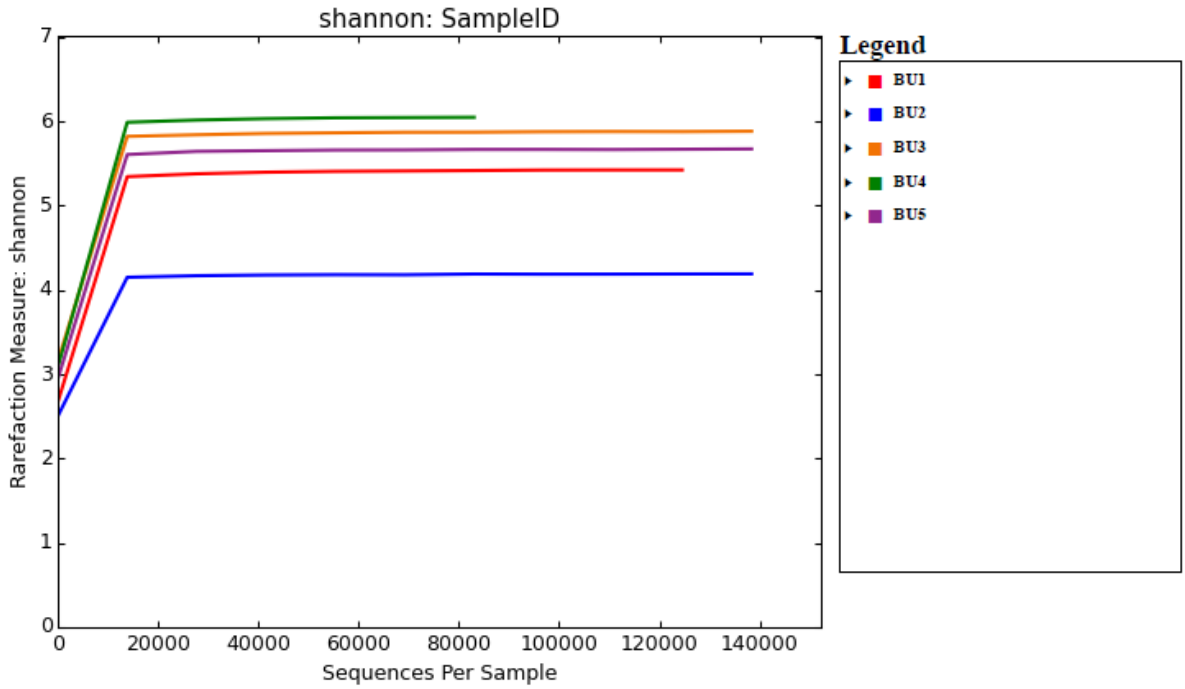

Figure 4.7: Rarefaction plot indicating the sequence coverage. (Butha-Buthe samples shown as a demonstration) ***Other samples shown in the appendices.**

From the results of relative bacterial abundance in all the samples analyzed, Firmicutes and Proteobacteria were the dominating phyla (fig. 4.8). There was also an observable decrease in Proteobacteria and an increase in Firmicutes as the fermentation was progressing. Proteobacteria is the major phylum of gram negative bacteria and they include a wide variety of human microbiota and soil-borne as well as pathogenic and spoilage microorganisms. As for Firmicutes they are the major group of gram positive bacteria and they include a wide variety of LAB. This group of bacteria usually produces by-products (i.e. bacteriocins, CO_2, organic acids) that will

inhibit gram negative bacteria (Mufandaedza *et al.*, 2006; Perez *et al.*, 2014; Rhee *et al.*, 2011; Todorov & Dicks, 2005). Hence as the fermentation is progressing, there is an evident decrease in Proteobacteria as the Firmicutes are increasing. The Proteobacteria in the samples is likely to come from the raw materials used to prepare the product as well as the hygiene of the producer. The Firmicutes are derived from the raw materials as well as from the starter cultures (Hammes *et al.*, 2005). It is a good and promising indication safety wise that there is a decrease in Proteobacteria as the fermentation is progressing. *Data sets on MK1 could not be obtained due to circumstances which could not be controlled.

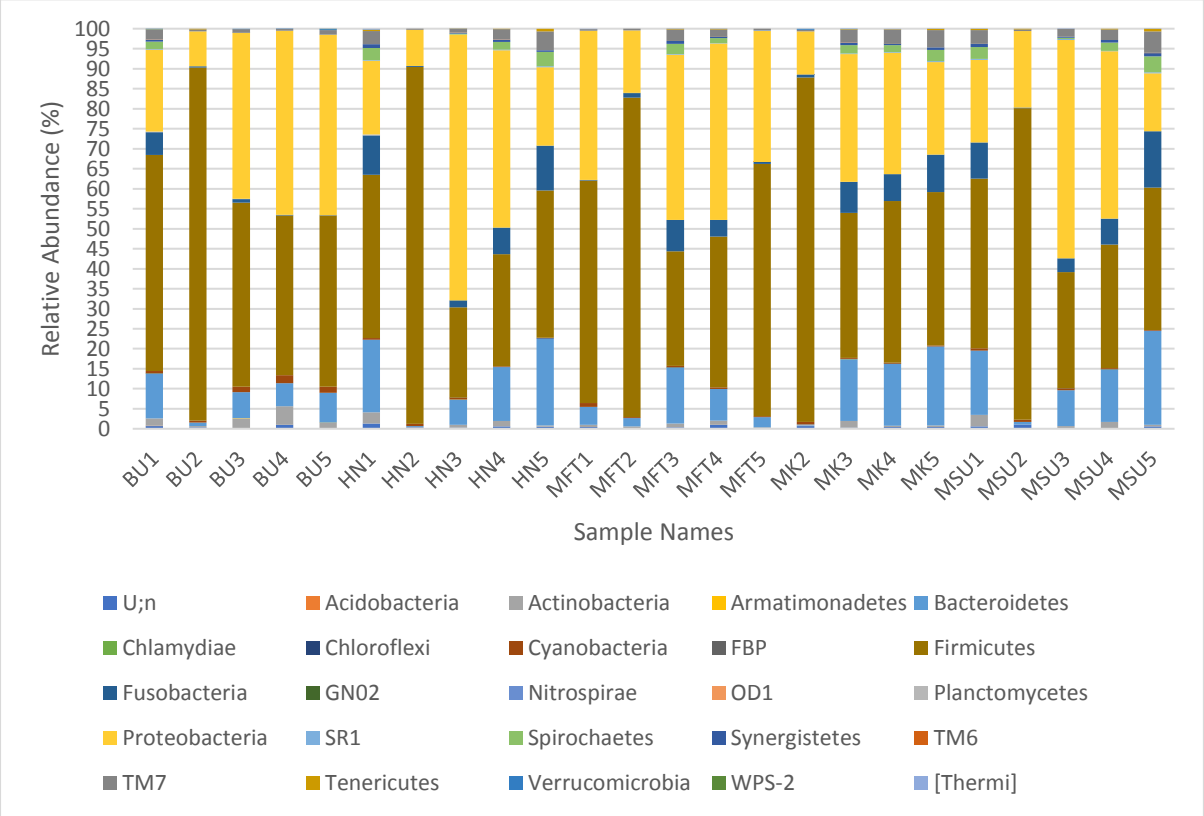

Figure 4.8: Beta-diversity of all *sesotho* samples during the fermentation process. (Phylum level)

4.4.4 Heat map analysis

The taxonomic heat map (fig. 4.9) showed that the patterns of abundance and diversity of the bacterial distribution (class level) across all samples is similar with the correlating stages of the process. Demonstrated by the heat map is that bacilli and Gammaproteobacteria were dominant across all samples. Bacilli class includes the LAB that notably play important roles such as preservation, flavor as well as nutritional benefits in fermented food (Alvarez-Sieiro *et al.*, 2016; Jeevaratnam *et al.*, 2005; Pundir *et al.*, 2013). Although the heat map analysis indicated Bacilli and Gammaproteobacteria as dominant across all samples, Bacilli dominated the 2nd stage more than other stages, a trend that was similar from all the brew sessions. Gammaproteobacteria is a class of several medically important bacterial groups. A number of important pathogens such as *Salmonella*, *Escherichia coli*, and *Vibrio* belong to this class (Cálix-Lara *et al.*, 2014; Kergourlay *et al.*, 2015; Mufandaedza *et al.*, 2006; Ben Omar & Ampe, 2000; Vuyst, 2004). Indicated from the heat map is that Gammaproteobacteria particularly dominated the 1st and mostly the 3rd sampled stages. It is at these stages where the brewers manually get involved as they add the starter cultures as well as some raw ingredients (i.e. starting raw materials in stage 1 and sorghum malt in stage 3). Some of the bacteria belonging to this class are soil-borne (sourced from the raw ingredients) whereas others are harbored from the brewers (as part of the normal human microbiota). It is interesting to note that the pattern of existence of the Gammaproteobacteria was similar in all the analyzed samples from Lesotho, despite the difference in brewers in different locations.

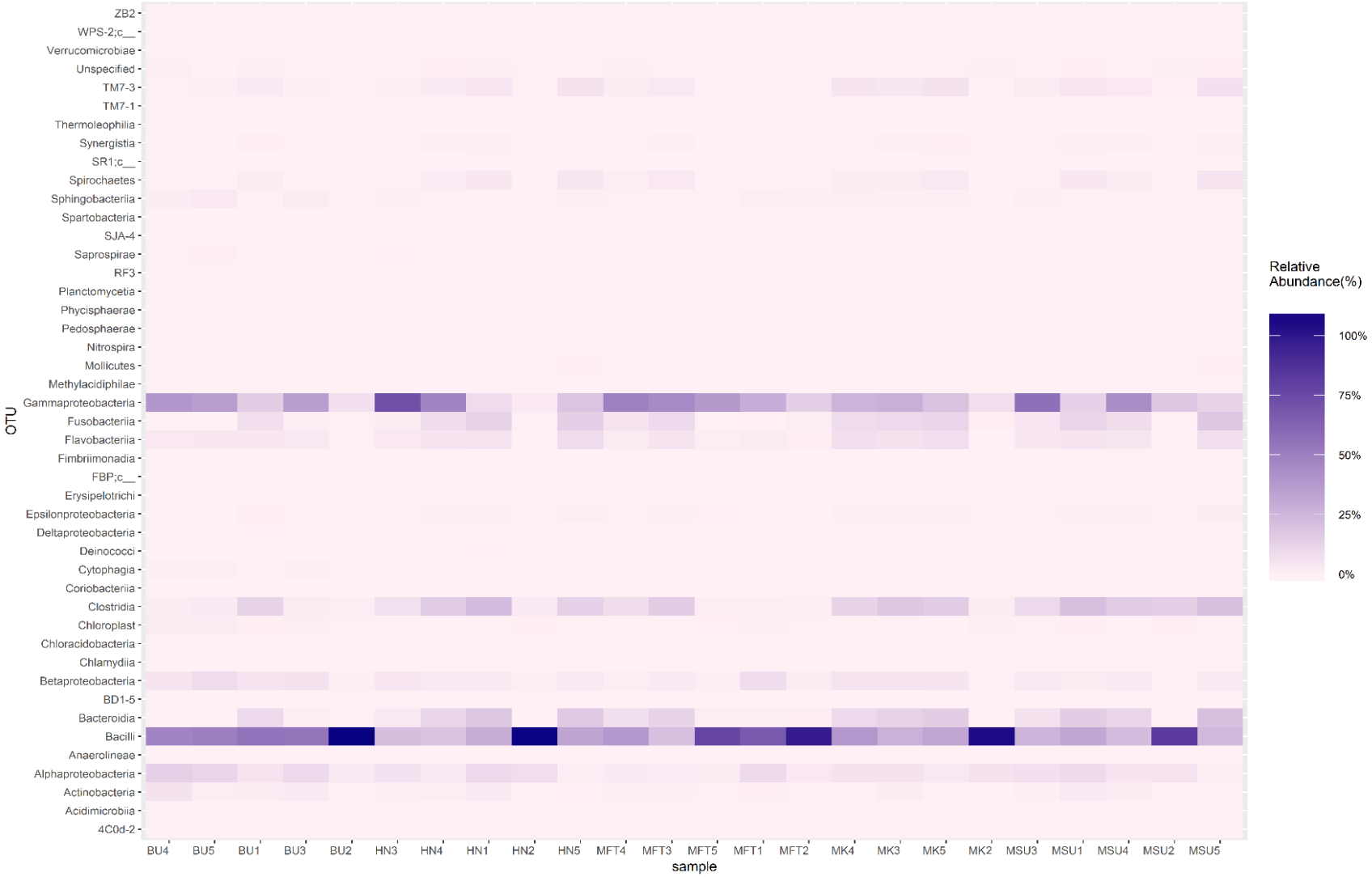

Figure 4.9: Heat map showing the relative abundance of bacteria in all *sesotho* samples (class level).

4.4.5 Geographical bacterial taxonomic distribution

4.4.5.1 Bacterial dynamics from the brew session at Maseru

From the bacterial dynamics in fermenting brews in Maseru (fig. 4.10), it was observed that as the fermentation started, there was not any bacterial group dominating, with bacterial groups belonging to classes of Gammaproteobacteria, Fusobacteria, Clostridia, Bacilli and Bacteroidia having a competitive share of dominance. As the fermentation progressed (2nd stage), there was an emergence of *Lactobacillus* (52%) as it dominated the stage and other bacterial groups belonging to the genera *Wiesella*, *Enterococcus Leuconostoc* and *Pediococcus* had a combined share of 19% and, Clostridium (10%) had a competitive share, bacterial groups belonging to the class of Gammaproteobacteria had a combined share of 13%. In the 3rd stage, there was an observable decline in *Lactobacillus* as several genera belonging to Gammproteobacteria were dominant. As the fermentation progressed, it was interesting to note that there was an observable increase in *Streptococcus* numbers and exhibiting a competitive share in bacterial composition of *sesotho*. It was also interesting to note that even though bacterial groups belonging to the class of Gammaproteobacteria were present, they were represented at low numbers in the final product.

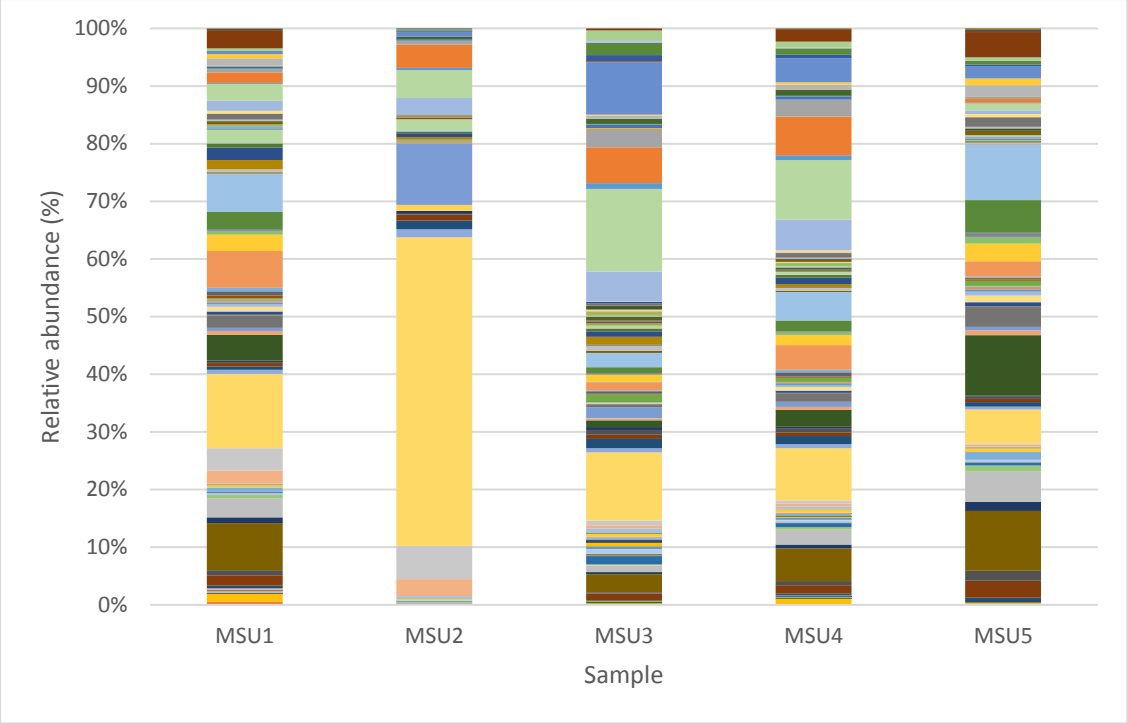

Figure 4.10: Beta-diversity of *sesotho* brewed at Maseru (genus level) (legends on all charts representing the bacterial dynamics is provised in the appendices)

4.4.5.2 Bacterial dynamics from the brew session at Mokhotlong

As the bacterial dynamics from the brew session at Mokhotlong were followed (fig. 4.11), there was an observable dominance in *Lactobacillus* (81 %) in the 2nd stage and bacterial groups belonging to the class of Gammaproteobacteria enjoying a combined share of approximately 15% as the fermentation was progressing. As it progressed to the 3rd stage, there was an observable decline in Firmicutes (44%) as several genera belonging to Gammaproteobacteria emerged (combined share of 35%), bacteria belonging to *Fusobacteria* enjoying a share of 9% as bacteria belonging to the class of Bacteroidia representing a share of 5% in abundance. It was at this stage where there was no obvious dominating bacteria at genus level. As the fermentation progressed, several bacterial groups belonging to the class of Bacilli (combined share of approximately 65 %) began to emerge and reclaiming dominance genera belonging to Gammaproteobacteria were seen to decline.

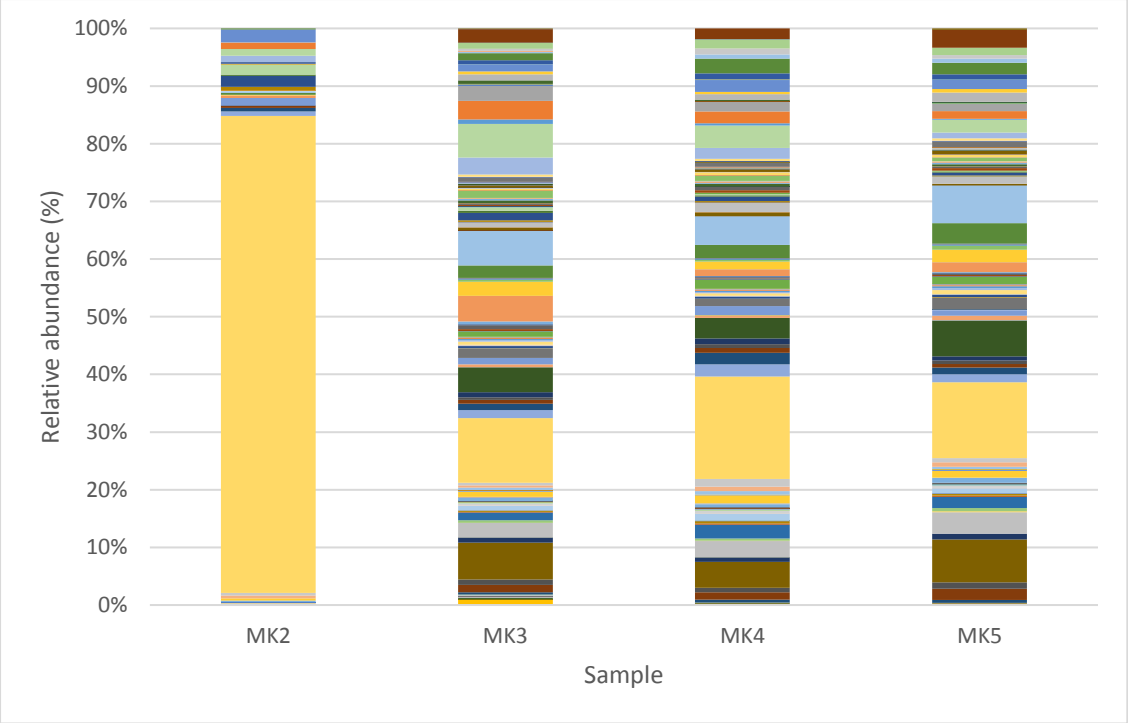

Figure 4.11: Beta-diversity of *sesotho* brewed at Mokhotlong (genus level).

4.4.5.3 Bacterial dynamics from the brew session at Mafeteng

Bacterial dynamics at the brew session at Mafeteng (fig. 4.12) exhibited *Lactobacillus* (44%) to dominate from the beginning of the fermentation and genera belonging to Gammaproteobacteria (25 %) also seemed to have competitive share in the beginning stages. As the process went on, *Lactobacillus* continued to dominate as there is an observable decrease in bacterial groups belonging to Gammaproteobacteria (9 %). However, it was during the 3rd stage where several genera that belong to Gammaproteobacteria, Clostridia, *Fusobacteria*, Bactreroidia and Bacilli were abundant. As the fermentation progressed there was an observable decrease in other bacterial groups and *Lactobacillus* (35%) dominated.Also in the brew session there was no significant share of *Streptococcus* and other genera belonging to the LABs but mostly *Lactobacillus*.

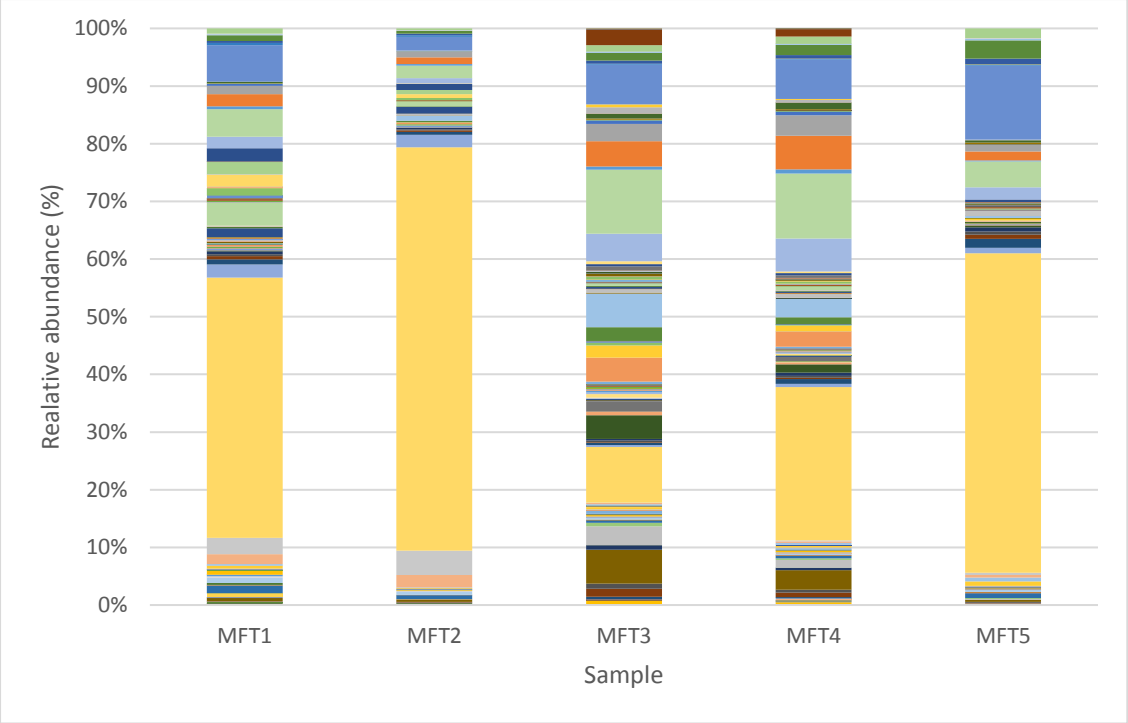

Figure 4.12: Beta-diversity of *sesotho* brewed at Mafeteng (genus level).

4.4.5.4 Bacterial dynamics from the brew session at Thaba-Tseka

The bacterial dynamics of brews at Thaba-Tseka (fig. 4.13) showed that at the initial stages of the brewing process there was no bacterial group dominating, with bacterial groups belonging to classes of Gammaproteobacteria, *Fusobacteria*, Clostridia, Bacillus and Bacteroidia had a competitive share of dominance. As the process progressed, *Lactobacillus* took an almost complete dominance (84 %), and as this happened, there was an observable decline in other bacterial groups. Nonetheless, in the 3rd stage there was no dominance observable as Gammaproteobacteria, *Fusobacteria*, Clostridia, Bacillus and Bacteroidia all had a competitive share. As the fermentation progressed, bacteria belonging to the genera of Gammaproteobacteria still enjoyed a good share of dominance. Interestingly, *Lactobacillus* alone did not dominate the final stages, but it was a combination of several genera from Firmicutes, Bacteroidetes and *Fusobacteria* sharing the dominating duties.

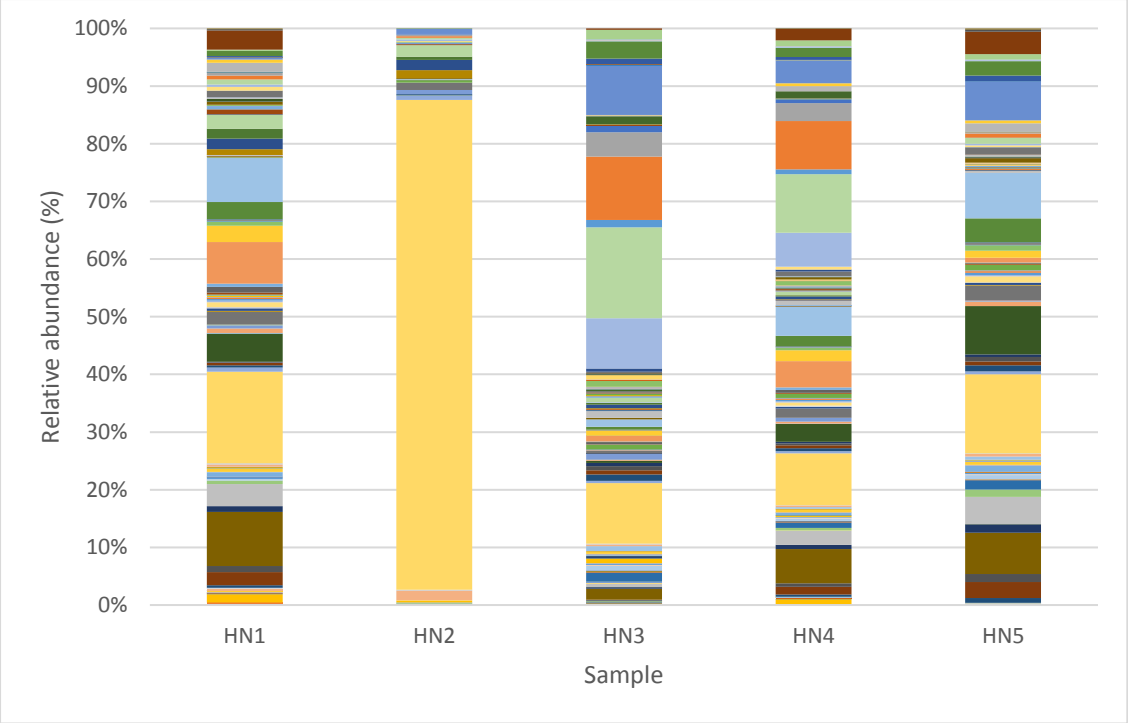

Figure 4.13: Beta-diversity of *sesotho* brewed at Thaba-Tseka (genus level).

4.4.5.5 Bacterial dynamics from the brew session at Butha-Buthe

As the bacterial dynamics for the brew session from Butha-Buthe were followed (fig.4.14), several genera belonging to Gammaproteobacteria and Bacillus competitive shares, however *Lactobacillus* (36%) was the dominant genus of all represented genera. As the process progressed, *Lactobacillus* (83%) took over to dominate as other bacterial groups such as Gammaproteobacteria were declining. As the process got in to the 3rd stage it is fascinating that *Lactobacillus* (26%) still took the dominating role and as the fermentation progressed on to the next stage, its dominance remained despite an emergence of genera such as *Pediococcus*, *Enterococcus*, *Wiesella* and *Leuconostoc* complementing the dominance of Firmicutes. It is interesting to note that as the fermentation was progressing, there was a decline in the diversity of Gammaproteobacteria.

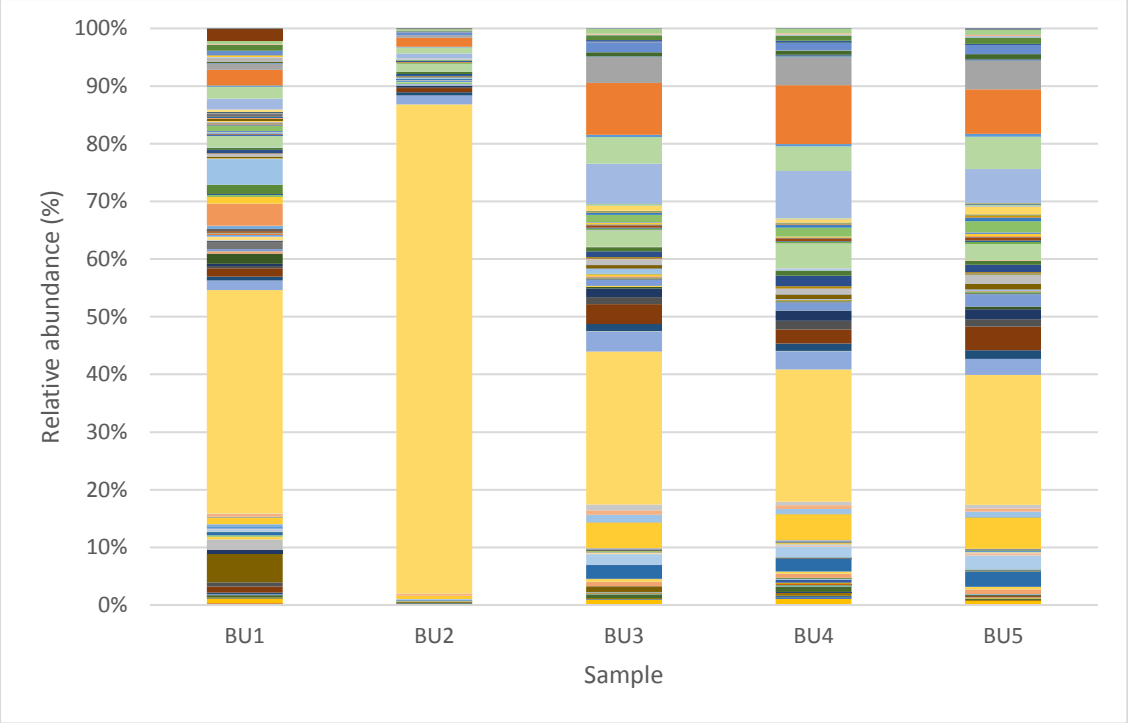

Figure 4.14: Beta-diversity of *sesotho* brewed at Butha-Buthe (Genus level).

4.5 Linking bacterial diversity with geographical difference, chemical profile and the brewer

In order to establish the clear consensual definition of the bacterial diversity of *sesotho*, batches from distinct geographical places by different brewers had to be followed. The bacterial diversity at different stages during the fermentation process is indicated on the PCoA (fig. 4.15). From the clustering on the PCoA plot, there are significant differences in diversities at stages 1, 3 and 4. A very slight difference in diversity in the 5th stage, between the different geographical locations was observed. Despite the geographical difference of breweries (fig. 4.1), the diversity in the 2nd stage was very similar and this stage was dominated by the LAB and this is also the stage that yielded more lactate production (fig. 4.16). From the PCoA analysis, it is clear that as much as there are regional differences in the bacterial composition, LAB (notably *Lactobacillus*) are of crucial importance in the consensual bacterial composition of beer.

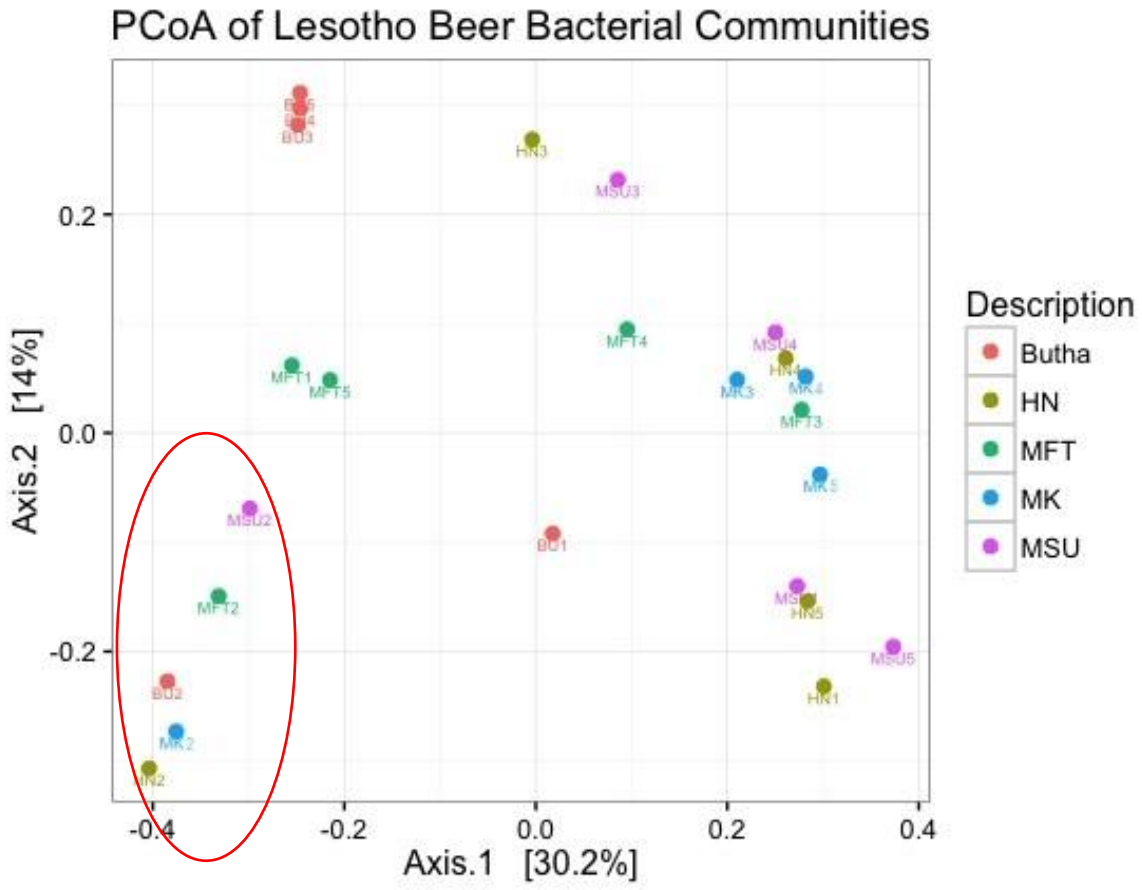

Figure 4.15: PCoA plot for *sesotho* bacterial communities.

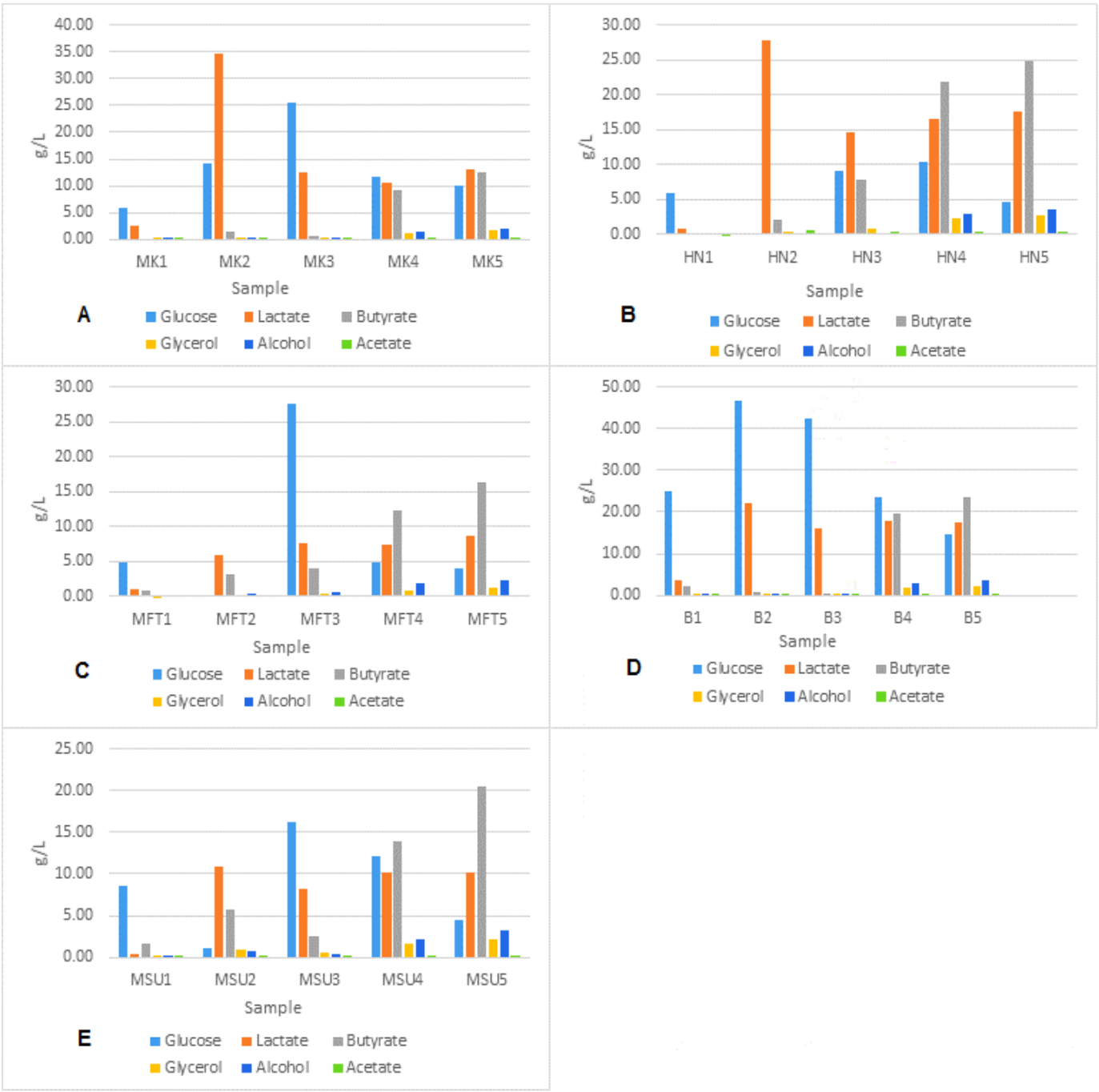

Figure 4.16: Chemical profiles during the respective brew sessions (A, Mafeteng) (B, Thaba-Tseka) (C, Mafeteng) (D, Butha-Buthe) (E, Maseru).

The chemical profiles of the respective fermentation sessions were determined and exhibited a general pattern, similar during each fermentation process (fig. 4.16). An observable production of lactate and butyrate was detected as the fermentation progressed. Literature indicated (Khalid, 2011) that both lactate and butyrate are produced by LAB, and from the bacterial dynamics we observed that LAB dominated the processes, hence the higher yields of butyrate and lactate. As for glucose, it

followed an immediate increase at the initial stages probably from the breakdown of flour and sorghum constituents and then an observable decrease as the fermentation approached the final stages. Glucose may also derive from the brewing style or recipe. Brewers in Butha-Buthe and Mafeteng had quite different recipes as compared to other brewers, including wheat flour as an additional ingredient. However, they added it at different stages, as brewers in Mafeteng added wheat during the 3rd stage, whereas those in Butha-Buthe from the 1st stage. The decrease in glucose towards the final stages was due to the yeast propagation as it was utilized to produce alcohol (explaining the production of alcohol in the final stages of the fermentation process) and the presence of LAB as well as other genera (Khalid, 2011; Marco *et al.*, 2017; Muller, 2003; De Vuyst & Neysens, 2005).

ADONIS (Table 4.1) analysis shows the impact of the factor on the bacterial diversity. From the ADONIS, lactate had 10% influence (at a significance of $p < 0.01$) on the bacterial diversity, which correlates with the observed LAB at stage 2 of production. Geographical location of the brewer or producer had about 28.9% (at a significance of $p < 0.005$) influence on the bacterial diversity, showing that location was the major determinant of bacterial diversity in the different beers.

Table 4.1: ADONIS analysis showing the impact of a factor on the bacterial diversity.

	F.Model	R2	p	
Lactate	2.6349	0.10696	0.008	p<0.01
pH	1.4631	0.06236	0.137	
Glucose	1.497	0.06371	0.117	
Butyrate	1.1512	0.04972	0.302	
Acetate	1.3365	0.05727	0.166	
Glycerol	1.3334	0.05715	0.16	
Brewery	1.9365	0.28962	0.005	P<0.01

The NMDS analysis (fig. 4.17) shows the correlation between the factor and the diversity during the fermentation process. From the NMDS, the length of the arrow indicates the strength of the correlation between the factor and the diversity. As can be seen from the NMDS, there seems to be a strong correlation between lactate concentration and the diversity abundances observed at the second stage. This was expected as this is the stage dominated by the LAB. Interestingly, the glucose concentration has a strong correlation with the diversity abundances observed in the later stages of the Butha-Buthe brewing process.

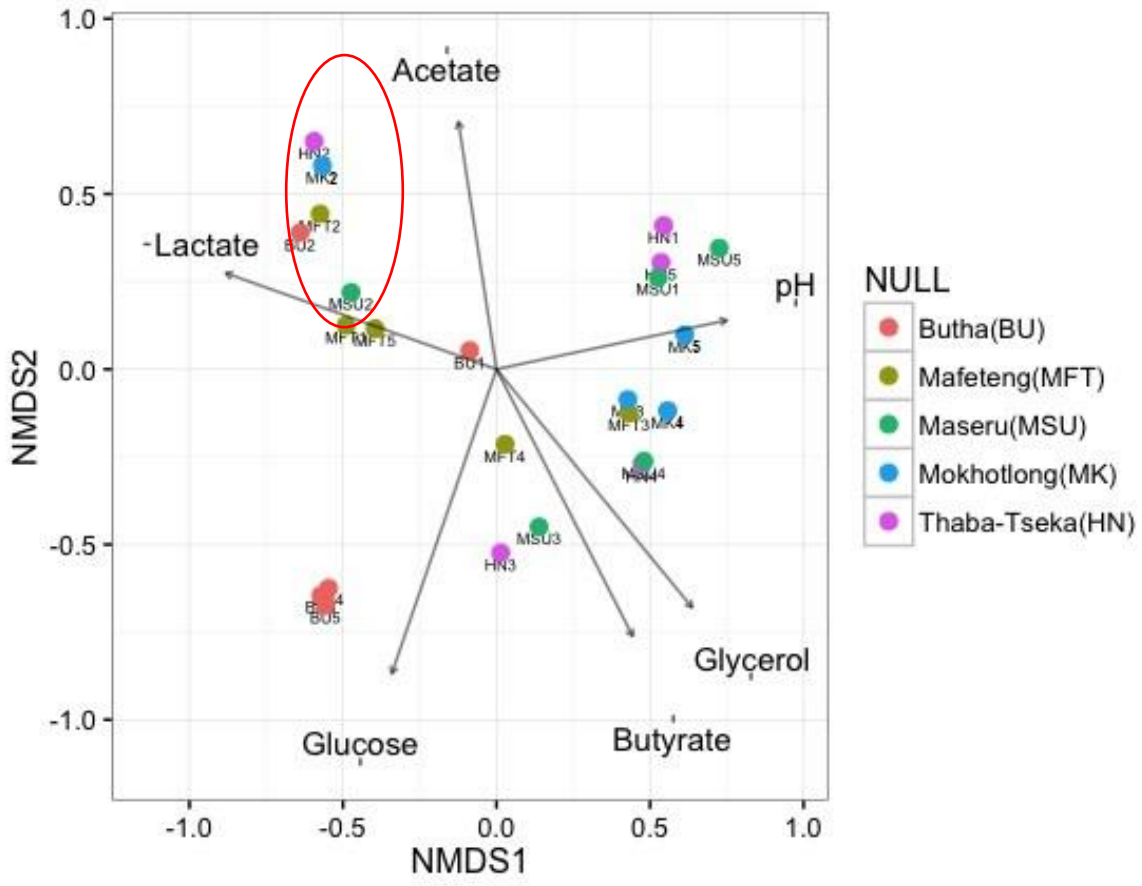

Figure 4.17: NMDS demonstrating the correlation of the factor with the diversity.

4.6 Diversity overview during the brewing process

In general, the results demonstrated that there was a shift in the bacterial diversity and distribution throughout the brewing process. The initial stage (1st stage) had a large bacterial diversity without any particular bacterial group displaying dominance. This was mainly due to the natural flora from raw materials, utensils and the starter culture (Chelule *et al.*, 2010; Marco *et al.*, 2017). As the fermentation was proceeding (2nd stage), there was a decline in other groups of bacteria as LAB dominated. It was at this stage where there was a high lactate yield (fig.4.16). Up to this stage the presence of enteric bacteria that were originally high in numbers seemed to have declined. In the 3rd stage there was an observable higher bacterial diversity, probably due to the addition of the second starter, sorghum malt and the manual manipulation by the brewer when mixing the opaque mixture (wort) with the sorghum malt and the starter. This stage demonstrated to be critical for hazard identification as it harbored potentially pathogenic bacteria in the form of enteric bacteria. However, as the fermentation progressed to the 4th stage, there seemed to be a decline in the huge diversity

observed in the 3rd stage, and it was in this stage where there were large yields of both lactate and ethanol (fig. 4.16). The decline in other bacterial groups maybe due to the presence ethanol as it can be toxic to most bacterial groups (Amenan *et al*., 2014; Bigot *et al.*, 2015; Kandler, 1983). In the 5th stage, the bacterial community was dominated by LAB and most of this bacterial group have been documented to confer some health benefits as probiotics (Table 4.2) (Mokoena *et al.*, 2016).

Table 4.2: Overview of selected LAB that confer some benefits (Adapted from (Mokoena *et al.*, 2016)).

LAB Strain	Benefit	Reference
Lactobacillus rhamnosus	Reduction of dental caries risk	(Nase *et al.*, 2001)
L. casei	Mitigation of obesity and prevention of diarrhoea	(Kechagia *et al.*, 2013)
Lactobacillus spp.	Lowering of cholesterol	(Anandharaj *et al.*, 2015)
L. reuteri; L. plantarum	Antifungal agents	(Ahmadova *et al.*, 2013)
L. brevis; L.casei	Immune system Modulation and mental health	(Shinde, 2012)
Lactobacillus spp.	Reduction of mycotoxins in fermented maize products	(Chelule *et al.*, 2010)

4.7 Putative ecological role of LAB and implications in cereal based fermented foods

Based on the results obtained, LAB was the major group of bacteria throughout the *sesotho* fermentation process. This is in agreement with several studies on the microbial dynamics of the fermented cereal based products (Assohoun-Djeni *et al.*, 2016; Giraffa, 2004; Hammes *et al.*, 2005; Katongole, 2008; Kunene *et al.*, 1999; Omar & Ampe, 2000; Ben Omar & Ampe, 2000). LAB belonging to genera such as *Lactobacillus, Enterococcus, Pediococcus, Leuconostoc,* and *Wiesella* were found to be dominant during the fermentation process. Extensive research on fermented food production has shown that these groups of bacteria have some benefits such as,

antimicrobial activity, probiotics, gastrointestinal disorders and cancer prevention (Table 4.2).

Specific antimicrobial mechanisms that LAB use are the production of organic acids, hydrogen peroxide, carbon dioxide, diacetyl and bacteriocins. The production of these antimicrobial compounds is of paramount importance as they inhibit other groups of bacteria and most importantly inhibit gram-negative and potentially pathogenic and spoilage groups of bacteria such as those belonging to the class of Gammaproteobacteria (Achi & Ukwuru, 2015; Benkerroum, 2013; Muller, 2003). Hence there was such a domination of LAB during the *sesotho* fermentation.

On the other hand, LAB (*Enterococcus* and *Lactobacillus*) are commonly used as probiotics around the world. Probiotics are defined as live microorganisms that when administered in appropriate amounts confer health benefits to the host (Fijan, 2014; Shori, 2015). It is important to note that *sesotho* harbored a great number of such important probiotic candidates. Health benefits conferred by probiotics include the reduction of cholesterol serum, enhancement of the immune response as well as the improvement of the intestinal health (German *et al.*, 1999; Oyedeji *et al.*, 2013; Rhee *et al.*, 2011; Taylor *et al.*, 2006). There is substantial evidence that support the use of probiotics in treating acute diarrheal diseases, prevention of the antibiotic-associated diarrhea as well as the improvement of lactose metabolism (Gadaga *et al.*, 2000; Kandler, 1983). Even though some of the health benefits are well documented, however further studies are needed to investigate undocumented benefits.

4.8 Conclusion

High throughput molecular techniques promised to be handy tools in exploring the microbial ecology of complex ecosystems such as those found in fermented foods. Application of culture independent molecular tools provide an unfiltered view of the microbial diversity as they also reveal those microbial groups that cannot be cultured (Bigot *et al.*, 2015; Kergourlay *et al.*, 2015). NGS was used to monitor the shifts in bacterial diversity and the distribution during the *sesotho* fermentation process. From the results obtained from this study, there were similarities in the microbial patterns in all the processes that were monitored across Lesotho. The study revealed various genera of LAB as the dominant group of bacteria during the *sesotho* fermentation process. Also demonstrated by the study was the safety efficacy of *sesotho*, as

microorganisms with the spoilage and pathogenic history declined to zero during the fermentation process. However, the study demonstrated that the hygiene of the brewer greatly contributed to the bacterial composition of the beer. As much as the bacterial dynamics demonstrated that there was a decline in Gammaproteobacteria, there are concerns about the safety of the beer as it does not only rely on the fermentation itself but also on the raw materials, utensils and hygiene of the brewer.

Interestingly, there was a consensus in the bacterial diversity of *sesotho* as the diversity in all the processes were similar despite the different geographical locations in which the fermentation processes were carried out. The chemical dynamics followed similar patterns in all brewing sessions, however the chemical profile did not appear to have great impact on the bacterial diversity as it only influenced 10% of the diversity. The study as demonstrated that the brewer (Mainly the back-slope starters) had 28% influence to the bacterial diversity.

This study has provided a clear picture regarding the shift in bacterial diversity during the *sesotho* fermentation. However, since it has been shown that there is an exchange in dominance in LAB and yeasts (Chapter 3), this calls for further investigations on the metagenomics insight of yeasts during *sesotho* fermentation. Further studies regarding the metabolic networks during fermentation process will be handy as they will aid in deeper studies of fermentation such as investigating the metabolic networks for flavor and aroma development as well as investigating thorough probiotic and nutritional potential of the process.

4.9 References

Achi, O. K. (2005). The potential for upgrading traditional fermented foods through biotechnology. *African J Biotechnol* **4**, 375–380.

Achi, O. K. & Ukwuru, M. (2015). Cereal-Based Fermented Foods of Africa as Functional Foods. *Int J Microbiol Appl* **2**, 71–83.

Ahmad, V., Khan, M. S., Jamal, Q. M. S., Alzohairy, M. A., Al Karaawi, M. A. & Siddiqui, M. U. (2016). Antimicrobial potential of bacteriocins: in therapy, agriculture and food preservation. *Int J Antimicrob Agents* **49**, 1–11.

Ahmadova, A., Todorov, S. D., Hadji-Sfaxi, I., Choiset, Y., Rabesona, H., Messaoudi, S., Kuliyev, A., Dora Gombossy de Melo Franco, B., Chobert, J. M. & Haertle, T. (2013). Antimicrobial and antifungal activities of Lactobacillus curvatus strain isolated from homemade Azerbaijani cheese. *Anaerobe* **20**, 42–49.

Alvarez-Sieiro, P., Montalbán-López, M., Mu, D. & Kuipers, O. P. (2016). Bacteriocins of lactic acid bacteria: extending the family. *Appl Microbiol Biotechnol* **100**, 2939–2951.

Amenan Anastasie Soro-Yao, Kouakou Brou, Georges Amani, P. T. and K. M. D. (2014). The use of LAB in starter cultures during the processing of fermented cereal based foods in west africa: a review. *Trop Life Sci Res* **25**, 1–10.

Anandharaj, M., Sivasankari, B., Santhanakaruppu, R., Manimaran, M., Rani, R. P. & Sivakumar, S. (2015). Determining the probiotic potential of cholesterol-reducing Lactobacillus and Weissella strains isolated from gherkins (fermented cucumber) and south Indian fermented koozh. *Res Microbiol* **166**, 428–439.

Assohoun-Djeni, N. M. C., Djeni, N. T., Messaoudi, S., Lhomme, E., Koussemon-Camara, M., Ouassa, T., Chobert, J. M., Onno, B. & Dousset, X. (2016). Biodiversity, dynamics and antimicrobial activity of lactic acid bacteria involved in the fermentation of maize flour for doklu production in Cote d'Ivoire. *Food Control* **62**, 397–404.

Benkerroum, N. (2013). Traditional Fermented Foods of North African Countries : Technology and Food Safety Challenges With Regard to Microbiological Risks. *Compr Rev food Sci food Saf* **12**, 54–89.

Bigot, C., Meile, J.-C., Remize, F. & Strub, C. (2015). Applications of Metagenomics to Fermented Foods. In *Fermented Foods, Part I (Biochemistry Biotechnol*, pp. 333–346. Edited by C. R. Didier, Montet; Ramesh. CRC Press 2015.

Cálix-Lara, T. F., Rajendran, M., Talcott, S. T., Smith, S. B., Miller, R. K., Castillo, A., Sturino, J. M. & Taylor, T. M. (2014). Inhibition of Escherichia coli O157: H7 and Salmonella enterica on spinach and identification of antimicrobial substances produced by a commercial Lactic Acid Bacteria food safety intervention. *Food Microbiol* **38**, 192–200.

Caplice, E. & Fitzgerald, G. F. (1999). Food fermentations: Role of microorganisms in food production and preservation. *Int J Food Microbiol* **50**, 131–149.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, K. & other authors. **(2011).** QIIME allows analysis of high-throuput community sequencing data. *Nat Methods* **7**, 335–336.

Cason, E. D. (2015). *Pathways for anaerobic hydrocarbon degradation in a nitrate reducing consortium*. University of the Free state.

Chelule, P. K., Mbongwa, H. P., Carries, S. & Gqaleni, N. (2010). Lactic acid fermentation improves the quality of amahewu, a traditional South African maize-based porridge. *Food Chem* **122**, 656–661.

Chuah, L. O., Shamila-Syuhada, A. K., Liong, M. T., Rosma, A., Thong, K. L. & Rusul, G. (2016). Physio-chemical, microbiological properties of tempoyak and molecular characterisation of lactic acid bacteria isolated from tempoyak. *Food Microbiol* **58**, 95–104.

Cocolin, L., Alessandria, V., Dolci, P., Gorra, R. & Rantsiou, K. (2013). Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. *Int J Food Microbiol* **167**, 29–43.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**, 2460–2461.

Escobar-Zepeda, A., Sanchez-Flores, A. & Quirasco Baruch, M. (2016). Metagenomic analysis of a Mexican ripened cheese reveals a unique complex microbiota. *Food Microbiol* **57**, 116–127.

Fijan, S. (2014). Microorganisms with claimed probiotic properties: An overview of recent literature. *Int J Environ Res Public Health* **11**, 4745–4767.

Gadaga, T. H., Mutukumira, A. N. & Narvhus, J. A. (2000). Enumeration and identification of yeasts isolated from Zimbabwean traditional fermented milk. *Int Dairy J* **10**, 459–466.

German, B., Schiffrin, E. J., Reniero, R., Mollet, B., Pfeifer, A. & Neeser, J. R. (1999). The development of functional foods: Lessons from the gut. *Trends Biotechnol* **17**, 492–499.

Giraffa, G. (2004). Studying the dynamics of microbial populations during food fermentation. *FEMS Microbiol Rev* **28**, 251–260.

Hammes, W. P., Brandt, M. J., Francis, K. L., Rosenheim, J., Seitter, M. F. H. & Vogelmann, S. A. (2005). Microbial ecology of cereal fermentations. *Trends Food Sci Technol* **16**, 4–11.

Hwanhlem, N., Chobert, J.-M. & H-Kittikun, A. (2014). Bacteriocin-producing lactic acid bacteria isolated from mangrove forests in southern Thailand as potential bio-control agents in food: Isolation, screening and optimization. *Food Control* **41**, 202–211.

Jeevaratnam, K., Jamuna, M. & Bawa, A. S. (2005). Biological preservation of foods - Bacteriocins of lactic acid bacteria. *Indian J Biotechnol* **4**, 446–454.

Kandler, O. (1983). Carbohydrate metabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek* **49**, 209–224.

Katongole, J. (2008). *The Microbial Succession in Indigenous Fermented Maize Products*. University of the Free State.

Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N. & Fakiri, E. M. (2013). Health benefits of probiotics: a review. *ISRN Nutr* **2013**, 1–7.

Kergourlay, G., Taminiau, B., Daube, G. & Champomier Vergès, M. C. (2015). Metagenomic insights into the dynamics of microbial communities in food. *Int J Food Microbiol* **213**, 31–39.

Khalid, K. (2011). An overview of lactic acid bacteria. *Int J Biosci* **1**, 2222–5234.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. & Glockner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res* **41**, 1–11.

Kunene, N. F., Hastings, J. W. & Holy, A. Von. **(1999).** Bacterial populations associated with a sorghum-based fermented weaning cereal. *Int J Food Microbiol* **49**, 75–83.

Marco, M. L., Heeney, D., Binda, S., Cifelli, C. J., Cotter, P. D., Foligne, B., Kort, R., Pasin, G., Pihlanto, A. & other authors. **(2017).** Health benefits of fermented foods : microbiota and beyond Michael Ga. *Curr Opin Biotechnol* 94–102.

Marshall, E. & Mejia-Lorio, D. J. (2012). Traditional fermented food and beverages for improved livelihoods. *Diversif Bookl*.

Martinez, R. C. R., Staliano, C. D., Vieira, A. D. S., Villarreal, M. L. M., Todorov, S. D., Saad, S. M. I. & Franco, B. D. G. de M. (2015). Bacteriocin production and inhibition of Listeria monocytogenes by Lactobacillus sakei subsp. sakei 2a in a potentially synbiotic cheese spread. *Food Microbiol* **48**, 143–152.

Masha, G. G. K., Ipsen, R., Petersen, M. A. & Jakobsen, M. (1998). Microbiological , rheological and aromatic characteristics of fermented Uji (an East African Sour Porridge). *World J Microbiol Biotechnol* **14**, 451–456.

McMurdie, P. J. & Holmes, S. (2013). Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS One* **8**, 1–11.

Minervini, F., De Angelis, M., Di Cagno, R. & Gobbetti, M. (2014). Ecological parameters influencing microbial diversity and stability of traditional sourdough. *Int J Food Microbiol* **171**, 136–146.

Mokoena, M. P., Mutanda, T. & Olaniran, A. O. (2016). African traditional fermented foods and beverages. *Food Nutr Res* **1**, 1–12.

Morozova, O. & Marra, M. A. (2008). Genomics Applications of next-generation sequencing technologies in functional genomics. *Genomics* **92**, 255–264.

Mozzi, F., Ortiz, M. E., Bleckwedel, J., De Vuyst, L. & Pescuma, M. (2013). Metabolomics as a tool for the comprehensive understanding of fermented and functional foods with lactic acid bacteria. *Food Res Int* **54**, 1152–1161.

Mufandaedza, J., Viljoen, B. C., Feresu, S. B. & Gadaga, T. H. (2006). Antimicrobial properties of lactic acid bacteria and yeast-LAB cultures isolated from traditional fermented milk against pathogenic Escherichia coli and Salmonella enteritidis strains. *Int J Food Microbiol* **108**, 147–152.

Muller, V. (2003). MINIREVIEW Energy Conservation in Acetogenic Bacteria. *Appl Environ Microbiol* **69**, 6345–6353.

Muyanja, C. M. B. K., Narvhus, J. A., Treimo, J. & Langsrud, T. (2003). Isolation, characterisation and identification of lactic acid bacteria from bushera: A Ugandan traditional fermented beverage. *Int J Food Microbiol* **80**, 201–210.

Nase, L., Hatakka, K., Savilahti, E., Saxelin, M., POnka, A., Korpela, R. & Meurman, J. . (2001). Effect of Long term consumption of a prbiotic bacterium, Lactobacillus rhamnosus GG, in milk on dental caries a caries risk in children. *Caries Res* **35**, 412–420.

Omar, N. & Ampe, F. (2000). Microbial community dynamics during production of the Mexican fermented maize dough pozol. *Appl Environ Microbiol* **66**, 3664–3673.

Oyedeji, O., Ogunbanwo, S. T. & Onilude, A. A. (2013). Predominant Lactic Acid Bacteria Involved in the Traditional Fermentation of Fufu and Ogi, Two Nigerian Fermented Food Products. *Food Nutr Sci* **4**, 40–46.

Park, E., Chun, J., Cha, C., Park, W., Ok, C. & Bae, J. (2012). Author ' s personal copy Bacterial community analysis during fermentation of ten representative kinds of kimchi with barcoded pyrosequencing Author ' s personal copy. *Food Microbiol* **30**, 197–204.

Paulson, J. N., Stine, O. C., Bravo, H. C. & Pop, M. (2013). Differential abundance analysis for microbial marker-gene surveys. *Nat Methods* **10**, 1200–1202.

Perez, R. H., Zendo, T. & Sonomoto, K. (2014). Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications. *Microb Cell Fact* **13**, S3.

Pundir, R. K., Rana, S., Kashyap, N. & Kaur, A. (2013). Probiotic potential of lactic acid bacteria isolated from food samples: An in vitro study. *J Appl Pharm Sci* **3**, 85–93.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res* **41**, 590–596.

Rhee, S., Lee, J.-E. & Lee, C.-H. (2011). Importance of lactic acid bacteria in Asian fermented foods. *Microb Cell Fact* **10**, S5.

Ruiz Rodríguez, L., Vera Pingitore, E., Rollan, G., Cocconcelli, P. S., Fontana, C., Saavedra, L., Vignolo, G. & Hebert, E. M. (2016). Biodiversity and technological-functional potential of lactic acid bacteria isolated from spontaneously fermented quinoa sourdoughs. *J Appl Microbiol* **120**, 1289–1301.

Schmieder, R. & Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. *Bioinformatics* **27**, 863–864.

Shendure, J. & Ji, H. (2008). Next-generation DNA sequencing. *Nat Biotechnol* **26**, 1135–1145.

Shinde, P. B. (2012). Probiotic: An overview for selction and evaluation. *Int J Pharm Pharm Sci* **4**, 14–21.

Shori, A. B. (2015). The potential applications of probiotics on dairy and non-dairy foods focusing on viability during storage. *Biocatal Agric Biotechnol* **4**, 423–431.

Steinkraus, H. K. (2002). Fermentations in world food processing. *Compr Rev food Sci food Saf* **1**, 23–32.

Taylor, J. R. N., Schober, T. J. & Bean, S. R. (2006). Novel food and non-food uses for sorghum and millets. *J Cereal Sci* **44**, 252–271.

Thairu, Y., Usman, Y. & Nasir, I. (2014). Laboratory perspective of gram staining and its significance in investigations of infectious diseases. *Sub-Saharan African J Med* **1**, 168–174.

Tikhonov, M., Leach, R. W. & Wingreen, N. S. (2013). Interpreting 16S metagenomic data without clustering separate sequence similarity from ecological similarity. *Int Soc Microb Ecol* **9**, 68–80.

Todorov, S. D. & Dicks, L. M. T. (2005). Pediocin ST18, an anti-listerial bacteriocin produced by Pediococcus pentosaceus ST18 isolated from boza, a traditional cereal beverage from Bulgaria. *Process Biochem* **40**, 365–370.

Vera-Pingitore, E., Jimenez, M. E., Dallagnol, A., Belfiore, C., Fontana, C., Fontana, P., von Wright, A., Vignolo, G. & Plumed-Ferrer, C. (2016). Screening and characterization of potential probiotic and starter bacteria for plant fermentations. *LWT - Food Sci Technol* **71**, 288–294.

Vuyst, L. De. **(2004).** Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci Technol* **15**, 67–78.

De Vuyst, L. & Neysens, P. (2005). The sourdough microflora: Biodiversity and metabolic interactions. *Trends Food Sci Technol* **16**, 43–56.

Ye, Q., Liu, J., Du, J. & Zhang, J. (2016). Bacterial diversity in submarine groundwater along the coasts of the yellow sea. *Front Microbiol* **6**, 1–14.

Yi-Seul Kim, Min-Cheol Kim, Soon-Jin Kim, In-Cheol Park, J.-O. and H.-Y. W. (2011). Analyses of bacterial communities in Meju, a Korean traditional fermented soybean bricks, by cultivation-based and pyrosequencing methods. *J Microbiol* **49**, 340–348.

Zhang, J., Kobert, K., Flouri, T. & Stamatakis, A. (2014). PEAR: A fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* **30**, 614–620.

Chapter 5

Scanning electron microscopic analysis of the biofilm on the earthen ware pot used to prepare sesotho

5.1 Abstract

It is a common practise in the villages to repeatedly use specific fermenting vessels especially in households that produce fermented beer for purchasing. This practise is instigated by the belief that these specific vessels aid in accelerating the fermentation rate as well as maintaining flavour consistency of the beer. It is thus assumed that the biofilm on the fermenting vessel serves as inoculate for the next batch. Extensive research in the fermentation fraternity has indicated that these specific vessels serve a duty similar to back-slopping. Three categories of fermenting vessels were used for the purpose of this study, the pot **(1)** that had only been used once, the pot **(2)** that was previously used to prepare *sesotho* and it had, 3 months since the cessation of preparing *sesotho* in it and the pot **(3)** that has been frequently used and was still in full use when taken for biofilm visualisations. The interior surfaces of all three pots were examined with the aid of the scanning electron microscopy (SEM). The main aim of this study was to find out whether biofilm formation takes place within such vessels and if that is the case, to establish which microorganisms are involved as well as their spatial arrangements. The micrographs obtained confirmed that biofilm formation does occur and they revealed that both bacteria and yeasts were the dominant, if not the only microorganisms, within this particular biofilm. From the micrographs it was revealed that there was a close proximity between bacteria and yeasts thus adding strength to the suggestion of a symbiotic relationship between the two microbial groups especially during fermentation. Microbial enumerations revealed that more microbial loads were on the crevice areas than on flatter surfaces within the interior of such fermenting vessels. However, the study has demonstrated the need for further confirmatory biochemical tests to discriminate between artefacts, fermentation remnants and genuine biological structures despite the SEM's ability to offer excellent resolution with the capacity to image complex shapes.

Keywords: Sesotho, Biofilm, Back-slopping, Scanning Electron Microscope, Yeasts, Bacteria, Symbiotic relationship

5.2 Introduction

Fermentation of food substrates is a common practice to preserve food in developing countries especially at village level households where there is a lack of food safekeeping facilities such as refrigeration. In such areas it is a customary practise that specific pots/FV (fig. 5.1) are repeatedly used to ferment various fermented products. This practise is very common especially at village level beer-producing households as there is a belief that the repeated use of the same fermenting vessel from one batch to the next encourages the consistency in the flavour and taste of the product (oral explanation obtained from the brewers).

Several studies on the microbial ecology of fermented food products have suggested that during the fermentation process there are symbiotic relations that take place towards the production of the final product that contains desirable sensory properties (Altay *et al.*, 2013; Kergourlay *et al.*, 2015; Smid & Lacroix, 2013). With the current knowledge of biofilms, it is thus assumed that over the process of time, biofilm layers that have accumulated on the surfaces of these special FV contribute to the final product by serving as an inoculum from one batch to the next (Achi & Ukwuru, 2015; Gadaga *et al.*, 2013). Biofilms are defined as a well-organized consortia of microbial cells. These assemblages are surface-associated and are enclosed in an extracellular polymeric substance (EPS) (Kokare *et al.*, 2009). Biofilms are heterogeneous, containing micro-colonies of various bacterial cells encased in the EPS separated from each other by the interstitial channels that allow liquid, gas flow and dispersion of nutrients (Donlan, 2002). Nutrient levels may be higher on the surfaces than in bulk solutions because they adsorb to them, hence surfaces are important microbial habitats. As a result microbial numbers and activities are usually greater on surfaces than in water (Camilli, 2006; Donlan, 2002; Kokare *et al.*, 2009). The biofilms trap nutrients that are required for the growth of microbial populations and strengthen the biofilm surface attachment (Costerton, 1995; Katongole, 2008).

Studies on microbial ecology do not only encompass the presence and interaction of microorganisms within an environment, but also involves their spatial arrangements. With the aid of his simple microscope, Anton Van Leeuwenhoek first observed the consortia of microbial cells on the tooth surface, with thus he can be credited for the discovery of these microbial assemblages called biofilms (Donlan, 2002).

Nonetheless, detailed analysis and examinations of biofilms would have to await for the development of transmission and electron microscopic techniques which provide higher resolutions and magnifications (Donlan, 2002; Jones *et al.*, 1969). Over the last two decades much of the work relied on the SEM for biofilm examination of the microbial spatial arrangements within their environment as well as their characterisation. Based on the above mentioned attributes of electron microscopes, in this study the SEM was chosen to carry out the biofilm analyses with special emphasis on the presence and spatial arrangements of the dominant microorganisms (chapters 3 and 4) during *sesotho* fermentation on the earthen ware FVs used to prepare *sesotho* beer.

Figure 5.1: *Sesotho* brewer stirring an earthen ware fermenting vessel (FV).

5.3 Scanning electron microscopy (The Principle)

Electron microscopes have a greater magnification power than light microscopes, with the limit being 0.2 nm. There are two kinds of electron microscopes, the SEM which is useful for three dimensional viewing of specimen surfaces whereas the transmission electron microscope (TEM) is used to observe the internal structure of the specimen down to the molecular level.

The SEM constitutes of the electron gun (light source) situated at the lens column, the lens system has two condenser lenses that focuses a beam of electrons into a specific area in the lens aperture of the objective lens. The objective lens will then focus the beam of electrons on the specimen to obtain a focussed image. While using the SEM, the specimen is coated with a thin film of a heavy metal such as gold to make it electron conductive. A beam of electrons in the SEM are then directed down onto the specimen and scan across it. Electrons scattered by the metal are collected and then activate a viewing screen to create an image.

5.3.1 Fixation

Specimens must be fixed in order to prevent any chemical changes and to preserve the original form on the specimen. This is carried out by immersing the material to be examined into fixative solutions. Specimens are immersed in the solutions with the surface to be examined facing upwards. Aldehyde fixatives are used as primary fixatives, followed by an oxidative fixative as a secondary fixative.

5.3.2 Dehydration

Specimens must be dried prior to SEM examination. This is because of the high vacuum of the microscope that hampers the observation of water containing specimens. This dehydration step makes use of a special apparatus called the critical point dryer. This is the standard and recommended technique. A critical point dryer is used and the material is dried using pressurised liquid CO_2 (to replace the ethanol) at 37 °C. This is because drying specimens through evaporation of water destroys the ultrastructure of the cell and thus damaging the specimen.

5.4 Materials and methods

5.4.1 Scanning preparation

Material for scanning electron microscopic examination was fixed in 0.1M (pH 7.0) sodium phosphate-buffered glutardialdehyde (3%) for at least 3 hours, followed by 1 hour fixation in similarly buffered osmium tetroxide (1%). The material was dehydrated in a graded ethanol series (50%, 70% and 95% for 20 minutes in each phase followed by two changes of 100% for 1 hour in each phase). The material was dried using a critical point dryer (Tousimis, Maryland, U.S.A.). After drying, material was mounted on stubs (Cambridge pin type 10 mm) by epoxy glue and gold coated (±

60 nm) with a Bio-Rad sputter coater (United Kingdom). The specimens were examined with a Shimadzu SSX-550 scanning electron microscope (Kyoto, Japan).

5.4.2 The nature and sources of specimens

Earthenware pots used to ferment *sesotho* were collected from local beer producing households in Lesotho. Pots were divided in three categories, the pot that was previously used to prepare *sesotho* (**2**), three months since the cessation of preparing *sesotho*, a new pot **(1)** that had only been used once and the pot **(3)** that has been frequently used and was still in full use when taken for biofilm visualisations. Before the SEM visualisations, a fresh batch of *sesotho* fermentation was run in all pots to view the fresh live microbial communities.

5.4.3 Preparation of specimens (pieces of pots)

The protocol for specimen preparation was according to Glauert (1974).

5.4.4 Microbial enumerations on pot surfaces

Surfaces of broken pot pieces were scrapped off to get an indication of microbial loads on the surfaces. From the surface of each pot, 1 g of the biofilm material was homogenised with 9 ml of sterile peptone physiological salt solution (Oxoid, Basingstoke, UK) followed by appropriate dilutions as required. Microbial counts were performed (duplicates) for the isolation of yeasts, lactic acid bacteria (LAB), coliforms and total viable bacteria.

Enumerations of yeasts were obtained by the spread plate method on Rose Bengal Chloramphenicol Agar (RBCA) (Oxoid, Basingstoke, UK) and aerobically incubated at 25 °C for 3-5 days. LAB counts were obtained by spread plate method on de Man Rogosa and Sharpe (MRS) and M17 (Biolab, Midrand, RSA) selective media. Appropriate dilutions were plated and then incubated anaerobically (anaerobic flask (Oxoid) in the presence of a gas-generating kit (Anaerobic System BR0038B, Oxoid) at 30°C for 48 hours. As for coliform enumeration, isolates were obtained by pour plating on Violet Red Bile agar (VRBA) (Oxoid) and incubated at 37°C for 24 hours. Total aerobic mesophilic counts were obtained by pour plating on PCA and incubating at 30 °C for 24 hours.

5.5 Results

5.5.1 SEM micrographs

Illustrated in Figure 5.2 are micrographs taken from the crevice or the depression area of the **(1)**, **(2)** and **(3)** fermenting vessels respectively. This area was chosen over the flatter area because the flatter area is easily washed off and therefore high microbial loads are unlikely on a flatter area as compared to the crevice area of the pots (Table 5.1).

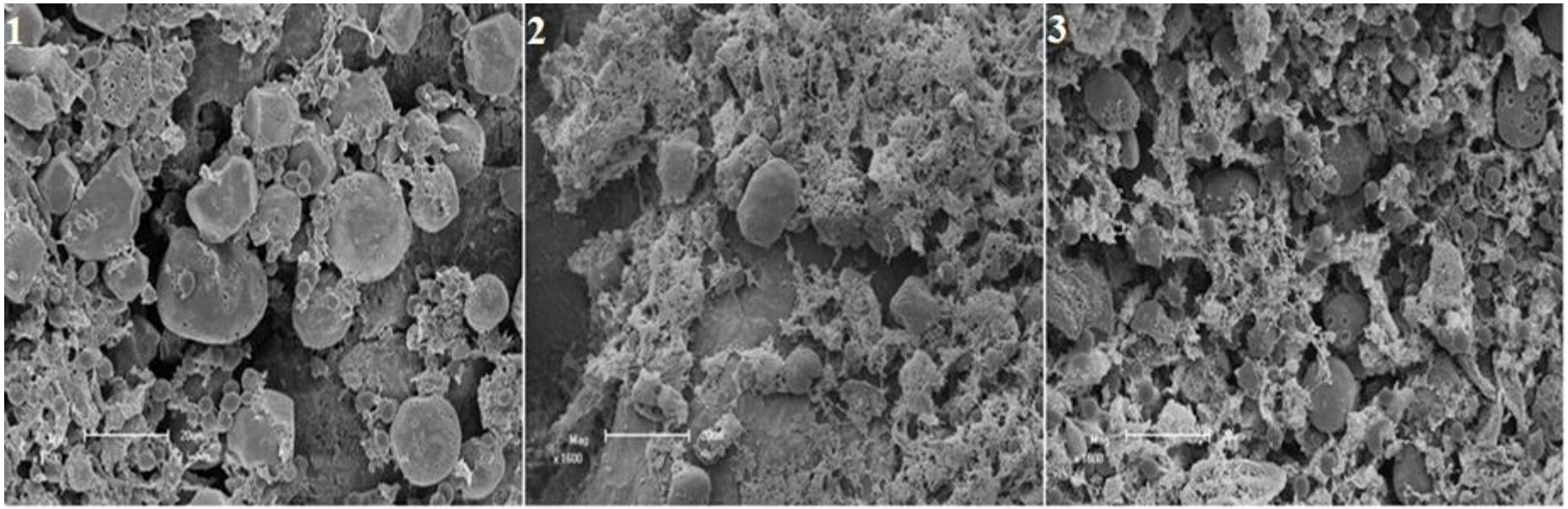

Figure 5.2: Micrographs taken from the crevice area of the Fermenting vessels (x 1600 magnification) (20 µm).

Figure 5.3 are close up magnifications of micrographs depicted in figs. 5.2 **(2)** and **(3)**, there seems to be more microbial loads and thread-like structures are also visible and this is not the case in Figure 5.2 **(1)**, and microbial loads seem to be less. Bacterial size range typically from 0.5 – 5 µm in length and in this image the cells seen are approximately 2 µm in length. The yeast cell size is approximately 4µm in diameter. However, yeast size can vary greatly depending on the species in question, typically measuring from 3 – 4 µm in diameter, even though some yeasts can reach sizes bigger than 40 µm. By virtue of this, from all pictures in figure 5.3, there are visible cocoon-like structures in which are suspected to be the remnants of fermentation (cereal grain particles) or clay-pot particles. The thread-like structures that are not observed in a vessel that had only been used once figure 5.2 **(1)** and are observed in Figures 5.33 **(2)** and **(3)** are suspected to be the EPS that had formed over time as this special vessels were repeatedly used.

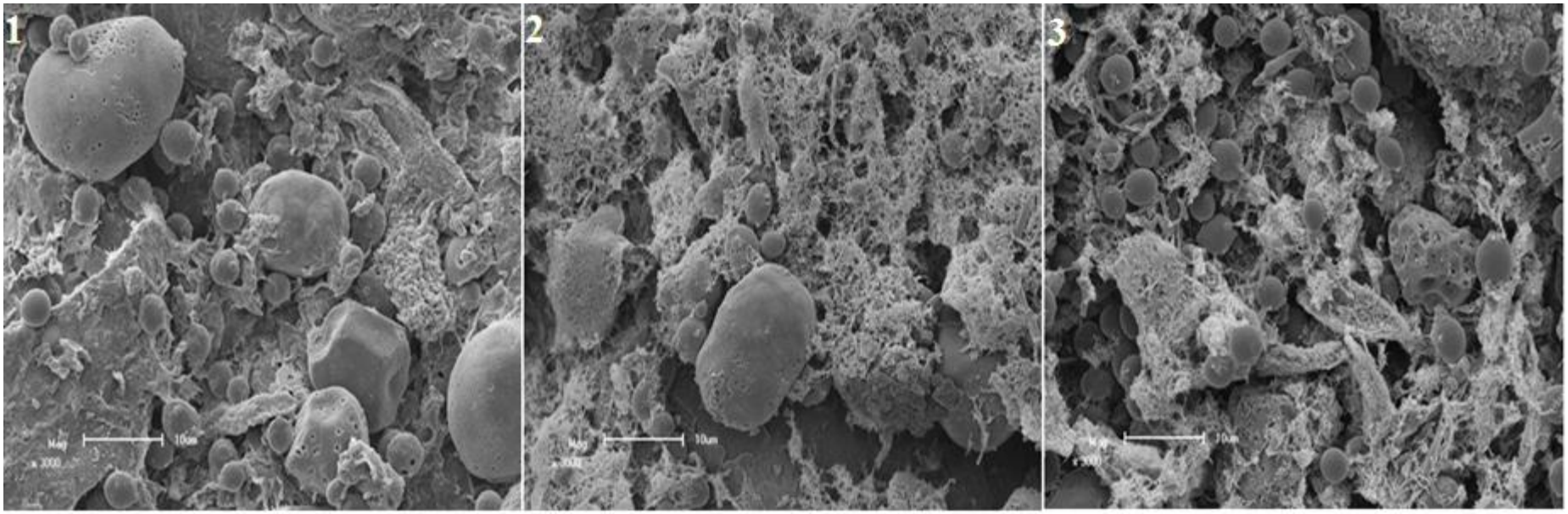

Figure 5.3: Close up magnifications of FV (x 3000 magnification) (10 µm).

Figure 5.4 are close up magnifications of micrographs depicted in Figure 5.3. From these larger images, it is interesting to note the close proximity of bacteria (bacterial rods) and yeasts, thus seemingly suggesting a symbiotic relationship which has been proposed by several researchers in the cereal fermentation fraternity (Giraffa, 2004; Holzapfel, 1997; Katongole, 2008; Narvhus & Henry, 2003; Oyedeji *et al.*, 2013; Vuyst, 2004; Watanabe *et al.*, 2008). From these set of images, we observed that there was a distinct attachment between bacteria and yeast cells. This spatial arrangement was a trend represented in all images. Different yeast morphologies can also be seen in these micrographs thus suggesting a wide yeast diversity during the *sesotho* fermentation. From these set of micrographs, differences in the biofilm layer microbial loads were clearly observable. Micrographs from pots **(2)** and **(3)** depicted higher microbial loads compared to pot **(1)**. This clearly supported the hypothesis that the vessels are indeed a seeding point of starter cultures, as these isolates remained viable in the biofilm of the pots for long periods.

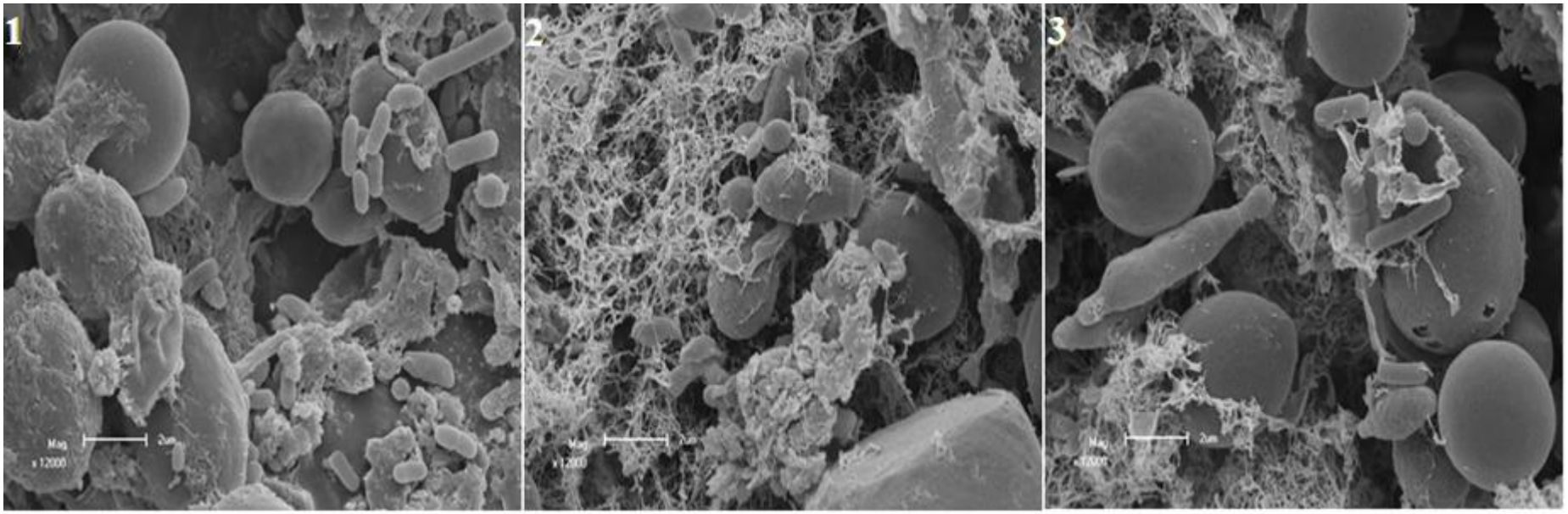

Figure 5.4: Close up magnifications (x 12 000 magnification) (2 µm).

5.5.2 Enumeration of microbial loads in the biofilms

Microbial counts (Table 5.1) indicated that the fermentation vessel that had been used several times carried a substantially higher microbial loads than a vessel that had only been used once. Enhanced microbial loads were found on the crevice areas as compared to flatter areas of the vessel. This may be due to the fact that isolates are easily washed off on the flatter areas than on the crevice areas where the isolates had a better survival rate based on improved attachment. As a result, biofilm development will appear over time. From the enumeration results obtained, it can be seen the dominating groups of microorganisms on the surface of the fermenting vessels were the LAB and yeasts.

Table 5.1. Microbial counts from biofilms developed on the pot surfaces **(*average counts of duplicates)**.

	Previously used pot			
	Counts (on media) (Log CFU/g)			
	VRBM	RBCA	M17	MRS
Crevice	5.41	7.78	6.45	8.43
Flat surface	TLTC	4.43	5.42	4.23
	Frequently used pot			
	Counts (on media) (Log CFU/g)			
	VRBM	RBCA	M17	MRS
Crevice	6.32	8.82	7.48	6.43
Flat surface	TLTC	5.65	6.21	5.89
	Pot only used once			
	Counts (on media) (Log CFU/g)			
	VRBM	RBCA	M17	MRS
Crevice	3.62	6.43	5.53	7.64
Flat surface	TLTC	2.43	3.28	5.54

5.6 Discussion

5.6.1 Biofilm formation on the pot surface

Fresh batches of *sesotho* beer were run on all three pots prior to SEM analysis. As stated earlier, the idea was to obtain fresh specimens which would give improved representative isolates of the microorganisms present on pot surfaces. A surface itself may serve as a nutrient source, such as a particle of an organic matter where microorganisms would adhere and catabolise the nutrients directly from the surface of the particle (Camilli, 2006; Wolfe & Dutton, 2015; Ye *et al.*, 2016). Micro-colonies naturally develop on such surfaces, thus leading to the formation of biofilms. Consequently it was expected to be observed on the pots, as confirmed by the results obtained by the SEM examination. Biofilms are defined as surface-associated microbial assemblages encased in an EPS developed over time (Costerton, 1995).

Several authors have indicated that cell to cell communication is of paramount importance towards the development and maintenance of the biofilm. They further indicated that the attachment of a cell to a surface is a signal for the expression of the biofilm genes. These genes encode proteins that synthesize cell to cell signalling molecules and begin polysaccharide formation (Camilli, 2006; Sturme *et al.*, 2002; Wai-Leung & Bassler, 2009). For example, *Pseudomonas aeruginosa*, an opportunistic pathogen which is also a very notorious biofilm former, releases signalling molecules called homoserine lactones (Eberl & Tümmler, 2004; Wai-Leung & Bassler, 2009). As these molecules are released, they accumulate and function as chemostatic agents to attract or recruit other *P. aeruginosa* cells initiating biofilm development. This process that mediates the bacterial cell-cell communication by secretion and detection of inducer molecules and serving the culture in monitoring population density is called quorum sensing (QS) (Smid & Lacroix, 2013).

From the SEM images close proximity of bacterial cells were observed on the pot surfaces, thick threads were also visible as well as some opaque irregular particles. Despite the SEM's ability to offer excellent resolution with the capacity to image complex shapes. There is a need for biochemical tests to confirm and discriminate between artefact and genuine structures.

5.6.2 Microbial interactions-Bacteria and yeast associations

From the close up images obtained, there was a close proximity observed between yeasts and the bacterial cells on the fermenting vessel surface. This observation further strengthens the suggestion of the symbiotic relationship that exists between bacteria and yeasts during cereal based fermentations. Extensive research on the microbial populations of the cereal based fermented products reported positive interactions between LAB and yeasts in different fermented food products (Katongole, 2008; Narvhus & Henry, 2003; Perez *et al.*, 2014; Smid & Lacroix, 2013; Vuyst, 2004; Watanabe *et al.*, 2008; Westby *et al.*, 1997; Zorba *et al.*, 2003). Further studies on this symbiotic relationships have indicated that yeasts stimulate the LAB providing compounds such as B-vitamins, pyruvate, succinate, propionate and CO_2. Also reports have shown that yeasts multiplication is associated with an increase in acid production/ formation by LAB in fermented products (Vollmar & Meuser, 1992). These acid-alcohol based symbiotic relationships occur when LAB are responsible for lowering of the pH due to the secretion of the organic acids allowing the yeasts populations to become competitive in the immediate environment (Blandino *et al.*, 2003).

5.7 Conclusions

Biofilms are heterogeneous, complex matrices containing micro-colonies of various bacterial cells encased in the EPS that is separated from each other by the interstitial channels that allow liquid, gas flow and dispersion of nutrients (Camilli, 2006; Kokare *et al.*, 2009). Extensive research has shown that the nutrient levels may be higher on the surfaces than in bulk solutions because they adsorb to them, hence surfaces serve as important microbial habitats. Biofilms that develop over time on the surfaces trap nutrients that are required for the growth of microbial populations and strengthens the biofilm surface attachment over time (Costerton, 1995; Donlan, 2002).

The repeated use of the same fermented vessel will develop into characteristic microbial population that will not be as diverse as that found in a new pot due to the competitive behaviour during biofilm development on the interior surface of such a vessel over time. SEM micrographs have shown that the biofilm contained the dominant microbiota (that have been reported in chapters 3 and 4) during fermentation

and as such, it will enable the succession of the indigenous fermentation, similar to what happens during back-slopping.

The study has demonstrated the need for further confirmatory biochemical tests to discriminate between artefacts, fermentation remnants and genuine biological structures despite the SEM's ability to offer excellent resolution with the capacity to image complex shapes.

5.8 References

Achi, O. K. & Ukwuru, M. (2015). Cereal-Based Fermented Foods of Africa as Functional Foods. *Int J Microbiol Appl* **2**, 71–83.

Altay, F., Karbancioglu-Güler, F., Daskaya-Dikmen, C. & Heperkan, D. (2013). A review on traditional Turkish fermented non-alcoholic beverages: Microbiota, fermentation process and quality characteristics. *Int J Food Microbiol* **167**, 44–56.

Blandino, A., Al-Aseeri, M. E., Pandiella, S. S., Cantero, D. & Webb, C. (2003). Cereal-based fermented foods and beverages. *Food Res Int* **36**, 527–543.

Camilli, A. (2006). Bacterial Small-Molecule Signaling Pathways. *Science (80-)* **311**, 1113–1116.

Costerton, J. W. (1995). Overview of microbial biofilms. *J Ind Microbiol* **15**, 137–140.

Donlan, R. M. (2002). Biofilms: microbial life on surfaces. *Emerg Infect Dis* **8**, 881–890.

Eberl, L. & Tümmler, B. (2004). Pseudomonas aeruginosa and Burkholderia cepacia in cystic fibrosis: Genome evolution, interactions and adaptation. *Int J Med Microbiol* **294**, 123–131.

Gadaga, T. H., Lehohla, M. & Ntuli, V. (2013). Traditional Fermented Foods of Lesotho. *J Microbiol Biotechnol Food Sci* **2**, 2387–2391.

Giraffa, G. (2004). Studying the dynamics of microbial populations during food fermentation. *FEMS Microbiol Rev* **28**, 251–260.

Glauert, A.M. (1974). Fixation, dehydration and embedding of biological specimens. In: Glauert, A.M. (Ed.), *Practical Methods in electron microscopy* **3**, 122-127.

Holzapfel, W. (1997). Use of starter cultures in fermentation on a household scale. *Food Control* **8**, 241–258.

Jones, H. C., Roth, I. L. & Sanders 3rd. W.M., W. M. (1969). Electron microscopic study of a slime layer. *J Bacteriol* **99**, 316–325.

Katongole, J. (2008). *The Microbial Succession in Indigenous Fermented Maize Products*. University of the Free State.

Kergourlay, G., Taminiau, B., Daube, G. & Champomier Vergès, M. C. (2015). Metagenomic insights into the dynamics of microbial communities in food. *Int J Food Microbiol* **213**, 31–39.

Kokare, C. R., Chakraborty, S., Khopade, A. N. & Mahadik, K. R. (2009). Biofilm: Importance and applications. *Indian J Biotechnol* **8**, 159–168.

Narvhus, J. A. & Henry, T. (2003). The role of interaction between yeasts and lactic acid bacteria in African fermented milks : a review **86**, 51–60.

Oyedeji, O., Ogunbanwo, S. T. & Onilude, A. A. (2013). Predominant Lactic Acid Bacteria Involved in the Traditional Fermentation of Fufu and Ogi, Two Nigerian Fermented Food Products. *Food Nutr Sci* **4**, 40–46.

Perez, R. H., Zendo, T. & Sonomoto, K. (2014). Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications. *Microb Cell Fact* **13**, S3.

Smid, E. J. & Lacroix, C. (2013). Microbe-microbe interactions in mixed culture food fermentations. *Curr Opin Biotechnol* **24**, 148–154.

Sturme, M. H. J., Kleerebezem, M., Nakayama, J., Akkermans, A. D. L., Vaughan, E. E. & De Vos, W. M. (2002). Cell to cell communication by autoinducing peptides in gram-positive bacteria. *Antonie van Leeuwenhoek, Int J Gen Mol Microbiol* **81**, 233–243.

Vollmar, A. & Meuser, F. (1992). Influence of starter cultures consisting of lactic-acid bacteria and yeasts on the performance of a continuous sourdough fermenter. *Cereal Chem* **69**, 20–27.

Vuyst, L. De. **(2004).** Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci Technol* **15**, 67–78.

Wai-Leung, N. G. & Bassler, B. L. (2009). Bacterial quorum-sensing network architectures. *Annu Rev Genet* **43**, 197–222.

Watanabe, K., Fujimoto, Æ. J., Sasamoto, Æ. M. & Dugersuren, Æ. J. (2008). uctDiversity of lactic acid bacteria and yeasts in Airag and Tarag , traditional fermented milk prods of Mongolia. *World J Microbiol Biotechnol* **24**, 1313–1325.

Westby, A., Reilly, A. & Bainbridge, Z. (1997). Review of the effect of fermentation on naturally occurring toxins. *Food Control* **8**, 329–339.

Wolfe, B. E. & Dutton, R. J. (2015). Review Fermented Foods as Experimentally Tractable Microbial Ecosystems. *Cell* **161**, 49–55.

Ye, Q., Liu, J., Du, J. & Zhang, J. (2016). Bacterial diversity in submarine groundwater along the coasts of the yellow sea. *Front Microbiol* **6**, 1–14.

Zorba, M., Hancioglu, O., Genc, M., Karapinar, M. & Gulden, O. (2003). The use of starter cultures in the fermentation of boza , a traditional Turkish be v erage. *Process Biochem* **38**, 1405–1411.

Chapter 6

6.1 General discussion and conclusions

The study was conducted in five geographically different regions across Lesotho, namely Maseru, Thaba-Tseka, Mokhotlong, Mafeteng and Butha-Buthe. The primary objectives of this study were to document the vital indigenous knowledge of cereal based fermented foods in Lesotho as well as to investigate the microbial ecology of *sesotho*. The study started off with a brief literature on the overview of cereal based fermentation as a household technology for food preparation and preservation. The literature section included a rationale, essentials, benefits, drawbacks and microbiological hazards of cereal based fermentation.

Using classical methods, microbial dynamics as well quantifying of the dominant microbial groups during the fermentation process were investigated. From the results obtained, various groups of bacteria such as LAB, enteric bacteria (sually with a pathogenic and spoilage history) as well as yeasts were present at the beginning of the process and as the fermentation progressed (increase in lactate, ethanol and acidity), counts of the enteric bacteria declined to undetectable levels as LAB and yeasts dominated the process.The study revealed that the microbial dynamics in all five regions followed a similar pattern throughout the fermentation process. The decline in enteric bacteria and an increase in LAB were a successful indication as it promised good safety and a probiotic potential for *sesotho*. Spontaneous fermentations result in activities of complex microbial systems whose diversity remain underestimated as they are made of cultivable and uncultivable groups of microorganisms (Bigot *et al.*, 2015). As a consequence, classical taxonomic identification techniques are not representative of the microbial diversity of food fermentations as they can give a filtered view and potentially misleading information about the microbial consortia of fermented products (Cocolin *et al.*, 2013). To address this issue, NGS was used to investigate bacterial diversity as well as the distribution during the *sesotho* fermentation process. The investigation was carried out by following five fermentation processes that were carried out by five different brewers in five geographically different locations across Lesotho. The main idea was to attain a consensual comprehensive definition of the bacterial diversity during the fermentation process. Observations obtained from the study demonstrated that shifts in the

bacterial diversity followed similar dynamics despite all processes being carried out at different geographical locations and by different brewers. The study demonstrated that dominant yeasts were those belonging to the genera of *Pichia, Issatchenkia* and *Saccharomyces* and as for dominant bacterial groups in all the processes were LAB belonging to the genera *Enterococcus, Pediococcus*, *Lactobacillus*, *Leuconostoc* and W*iesella.* Another interesting observation was that as the fermentation processes progressed, a decline in the enteric bacteria to undetectable levels corresponded with a simultaneous increase in LAB numbers. This trend was observed in all the processes followed across Lesotho.

NGS demonstrated that the third stage during the preparation of *sesotho* is critical for hazard analysis. This was the stage which followed the addition of the second back slope starter (supposedly different from brewer to brewer) culture as well as the ground sorghum malt. Due to the lack of control during microbial seeding, it seemed to be the most critical stage as it harbored the largest microbial diversity of all stages. Representatives at this stage, were mostly enteric bacteria and other bacterial groups that are usually found on the human body as part of the normal microbiota (Cálix-Lara *et al.*, 2014; Delzenne *et al.*, 2011; Kergourlay *et al.*, 2015). This is the stage where the brewer manually manipulates the brewing process, and thus contributing to the microbial content of the product. However as the fermentation progressed, there was an observable decline of these undesired microbial populations which is an indication of the fermentation efficacy to control harmful organisms during *sesotho* production (Chuah *et al.*, 2016; Holzapfel, 2002; Motarjemi, 2002; Oliveira *et al.*, 2014). Statistical results obtained from this study demonstrated that the chemical profile did not significantly influence the bacterial diversity whereas the brewer appeared to influence about 28% of the bacterial diversity.

Microbial ecology does not only encompass the presence and interaction of microorganisms, but also involves their spatial arrangements within their environments (Camilli, 2006; Wolfe & Dutton, 2015). From the microbial dynamics there was an observable lack of antagonism between yeasts and LAB and thus suggesting a symbiotic relationship. Since it is customary in the villages to repeatedly use specific fermenting vessels, especially in households that produce fermented beer for commercial reasons, the supporting role between these groups may secure continuous good brews. Within brewers, there is the belief that the use of specific

vessels aid in accelerating the fermentation rate as well as maintaining consistency in the flavour profile of the beer. Therefore on the basis of repeated usage of the vessels it was of importance that the biofilms within the pots were examined as it exhibited the arrangement of the microbial ecology of *sesotho* (Kokare *et al.*, 2009; Wolfe & Dutton, 2015). The biofilms also revealed the starter culture development and diversity. The SEM was used to examine the biofilms on the fermenting vessels used to prepare *sesotho*. From the SEM results, there was an observable proximity between yeasts and bacterial rods (presumably LAB) and thus suggesting that there was a symbiotic relationship between yeasts and LAB and that they did form biofilm linkages (Marsh *et al.*, 2014).

In general, cereal based fermented foods remain an integral part of diet within African communities especially marginalised groups where they rely on fermentation as a preferred method for food preparation and preservation (Achi & Ukwuru, 2015; Achi, 2005). Benefits such as improved nutritional value, increased shelf-life and palatability suggest the importance of fermented foods (Selhub *et al.*, 2014). In addition, together with the microbial cultures associated with them, fermented foods promise to be great harbours of probiotics and this calls for further research. Finally, metagenomics have opened new horizons regarding the research scope on fermented foods.

Further open research horizons

- Metagenomics approach can be applied to provide insights into the genes, structure, and function of products from probiotic microorganisms (Kergourlay *et al.*, 2015).
- *All-Food-Seq*, is a new metagenomic approach that involves untargeted deep sequencing of total genomic DNA in foods. This approach will enable the possibility to identify species from all kingdoms of life in a foodstuff in question. Concurrently, this will also reveal new horizons for evaluating the ingredient composition as well as its microbial content (Ripp *et al.*, 2014).
- Multi-omic approach such as metatranscriptomics and metabolomics could be utilised to source valuable information regarding the estimations of bacterial to environment interactions within the foodstuff matrix. This multi-omic approach will be of crucial aid in the flavour profile development studies (van Hijum *et al.*, 2013).

6.2 References

Achi, O. K. (2005). The potential for upgrading traditional fermented foods through biotechnology. *African J Biotechnol* **4**, 375–380.

Achi, O. K. & Ukwuru, M. (2015). Cereal-Based Fermented Foods of Africa as Functional Foods. *Int J Microbiol Appl* **2**, 71–83.

Bigot, C., Meile, J.-C., Remize, F. & Strub, C. (2015). Applications of Metagenomics to Fermented Foods. In *Fermented Foods, Part I (Biochemistry Biotechnol*, pp. 333–346. Edited by C. R. Didier, Montet; Ramesh. CRC Press 2015.

Cálix-Lara, T. F., Rajendran, M., Talcott, S. T., Smith, S. B., Miller, R. K., Castillo, A., Sturino, J. M. & Taylor, T. M. (2014). Inhibition of Escherichia coli O157: H7 and Salmonella enterica on spinach and identification of antimicrobial substances produced by a commercial Lactic Acid Bacteria food safety intervention. *Food Microbiol* **38**, 192–200.

Camilli, A. (2006). Bacterial Small-Molecule Signaling Pathways. *Science (80-)* **311**, 1113–1116.

Chuah, L. O., Shamila-Syuhada, A. K., Liong, M. T., Rosma, A., Thong, K. L. & Rusul, G. (2016). Physio-chemical, microbiological properties of tempoyak and molecular characterisation of lactic acid bacteria isolated from tempoyak. *Food Microbiol* **58**, 95–104.

Cocolin, L., Alessandria, V., Dolci, P., Gorra, R. & Rantsiou, K. (2013). Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. *Int J Food Microbiol* **167**, 29–43.

Delzenne, N. M., Neyrinck, A. M. & Cani, P. D. (2011). Modulation of the gut microbiota by nutrients with prebiotic properties: consequences for host health in the context of obesity and metabolic syndrome. *Microb Cell Fact* **10**, S10.

Holzapfel, W. H. (2002). Appropriate starter culture technologies for small-scale fermentation in developing countries. *Int J Food Microbiol* **75**, 197–212.

Kergourlay, G., Taminiau, B., Daube, G. & Champomier Vergès, M. C. (2015). Metagenomic insights into the dynamics of microbial communities in food. *Int J Food Microbiol* **213**, 31–39.

Kokare, C. R., Chakraborty, S., Khopade, A. N. & Mahadik, K. R. (2009). Biofilm: Importance and applications. *Indian J Biotechnol* **8**, 159–168.

Marsh, A. J., Hill, C., Ross, R. P. & Cotter, P. D. (2014). Fermented beverages with potential : Past and future perspectives. *Trends Food Sci Technol* **38**, 113–124.

Motarjemi, Y. (2002). Impact of small scale fermentation technology on food safety in developing countries. *Int J Food Microbiol* **75**, 213–229.

Oliveira, P. M., Zannini, E. & Arendt, E. K. (2014) Cereal fungal infection, mycotoxins, and lactic acid bacteria mediated bioprotection: From crop farming to cereal products. *Food Microbiol* **37**, 78–95.

Ripp, F., Krombholz, C. F., Liu, Y., Weber, M., Schäfer, A., Schmidt, B., Köppel, R. & Hankeln, T. (2014). All-Food-Seq (AFS): a quantifiable screen for species in biological samples by deep DNA sequencing. *BMC Genomics* **15**, 693.

Selhub, E. M., Logan, A. C. & Bested, A. C. (2014). Fermented foods, microbiota, and mental health: ancient practice meets nutritional psychiatry. *J Physiol Anthropol* **33**, 2.

van Hijum, S. A. F. T., Vaughan, E. E. & Vogel, R. F. (2013). Application of state-of-art sequencing technologies to indigenous food fermentations. *Curr Opin Biotechnol* **24**, 178–186.

Wolfe, B. E. & Dutton, R. J. (2015). Review Fermented Foods as Experimentally Tractable Microbial Ecosystems. *Cell* **161**, 49–55.

Chapter 7

7.1 Summary

Indigenous fermented foods represent large portions of daily diets particularly within the marginalised low-income groups. Most of the fermentation processes are spontaneous and are driven by complex microbial communities which are sourced from raw materials, producer's handling, utensils and unstandardized indigenous starter cultures. These complex microbial communities influence the organoleptic quality, nutrient availability as well as the safety of the fermented product. In order to improve the nutritional and organoleptic quality as well as safety of these fermented products, it is imperative that the processes are systematically studied and documented, the ingredients should be quantified and preparation conditions required for a successful fermentation identified. The primary objectives of this study were to document the vital indigenous knowledge of cereal based fermented foods in Lesotho and investigate the microbial loads of *sesotho* as well as to assess the possible stages where contamination or even pathogenic microorganisms might be introduced.

The diversity of cereal based products in Lesotho ranges from non-alcoholic and alcoholic gruels to alcoholic beverages. In this study, both non-alcoholic and alcoholic gruel based beverages have been documented. Non-alcoholic gruels include *motoho*, *ntsoana-tsike*, and *tsoeu-koto*, these food products are consumed as weaning food for infants as well as thirst quenching beverages. Alcoholic beverages are represented by *sesotho*, *hopose*, *sekumukumu* and *tintana.* Also outlined in this study are factors that could negatively impact the quality of fermented final products and the health of ultimate consumers. These include the compromised hygiene of food handlers and equipment, unstandardized temperature for fermentation, cooking and storage and the quality of raw materials and starters used.

In assessing the microbial content, a total of 25 different *sesotho* fermentation processes were scientifically investigated from the initial stages to the final product, including five locally different processes in each region representing the south, north, east, west and central. Five sampling points during the processing were carefully selected in order to get well-defined representative microbial patterns during the fermentation process. From following the microbial dynamics, lactic acid bacteria (LAB) and yeasts emerged as the dominant groups as the fermentation was

progressing. A combination of conventional culture-dependent and high-throughput culture-independent (Next Generation Sequencing) techniques were employed to complement a comprehensive representation of the microbial content and succession of *sesotho*. The results revealed that dominant yeasts were those belonging to the genera of *Pichia, Issatchenkia* and *Saccharomyces*. As for dominant bacterial groups in all the processes were LAB belonging to the genera of *Enterococcus, Pediococcus*, *Lactobacillus*, *Leuconostoc* and W*iesella*. It was interesting to observe that as the fermentations progressed, there was a decline in the enteric bacteria to undetectable levels, thus suggesting a promise of a good safety record as well as a probiotic potential for *sesotho*. This trend was observed in all the processes followed across Lesotho.

Microbial assessments indicated that the third stage during the fermentation was critical for contamination or introduction of pathogenic microorganisms. Harbored in this stage, was mostly enteric bacteria and other bacterial groups that are usually found on human body as part of the normal microbiota.

From the microbial succession patterns, there was an observable lack of antagonism between yeasts and LAB and thus suggesting a symbiotic relationship. The Scanning Electron Microscope was employed to examine the spatial arrangements of these groups of microorganisms as well as the biofilm formation on the fermentation vessel. From the micrographs obtained, there was an observable proximity between the LAB and yeasts thus strengthening the suggestion of symbiosis during the fermentation process of *sesotho*.

Keywords: Fermented products, Lesotho, Microbial succession, Sesotho, Next Generation Sequencing, Scanning Electron Microscope, Symbiosis

Appendices

Chapter3

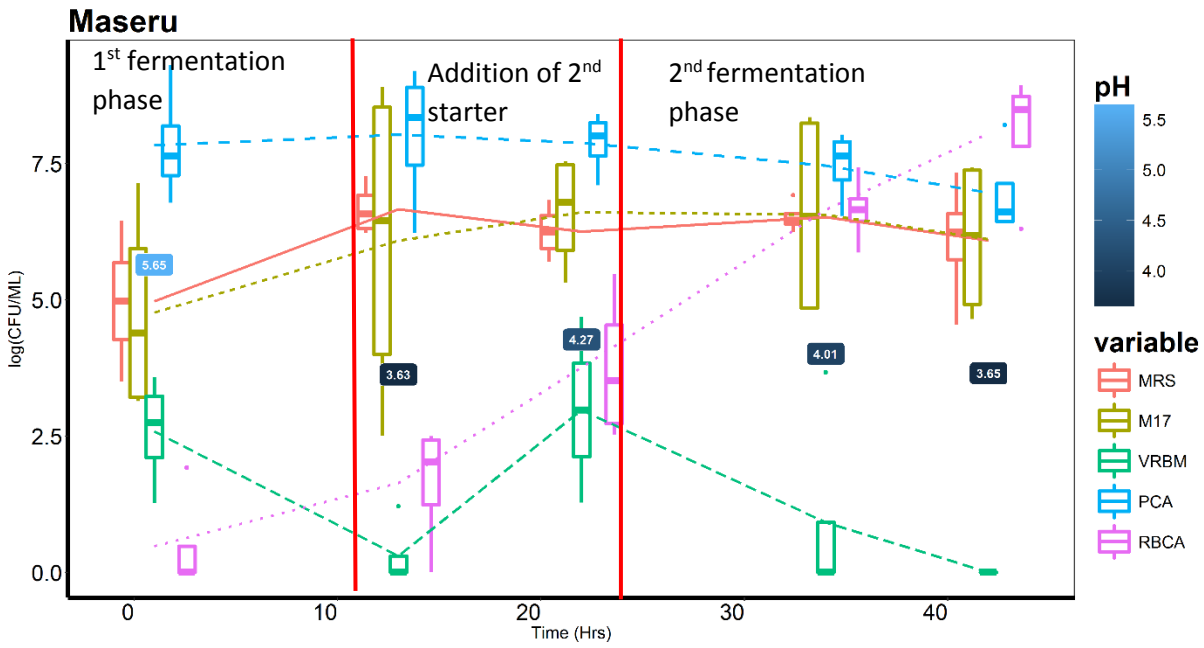

Figure 3A: Microbial patterns of *sesotho* during the fermentation processes in Maseru.

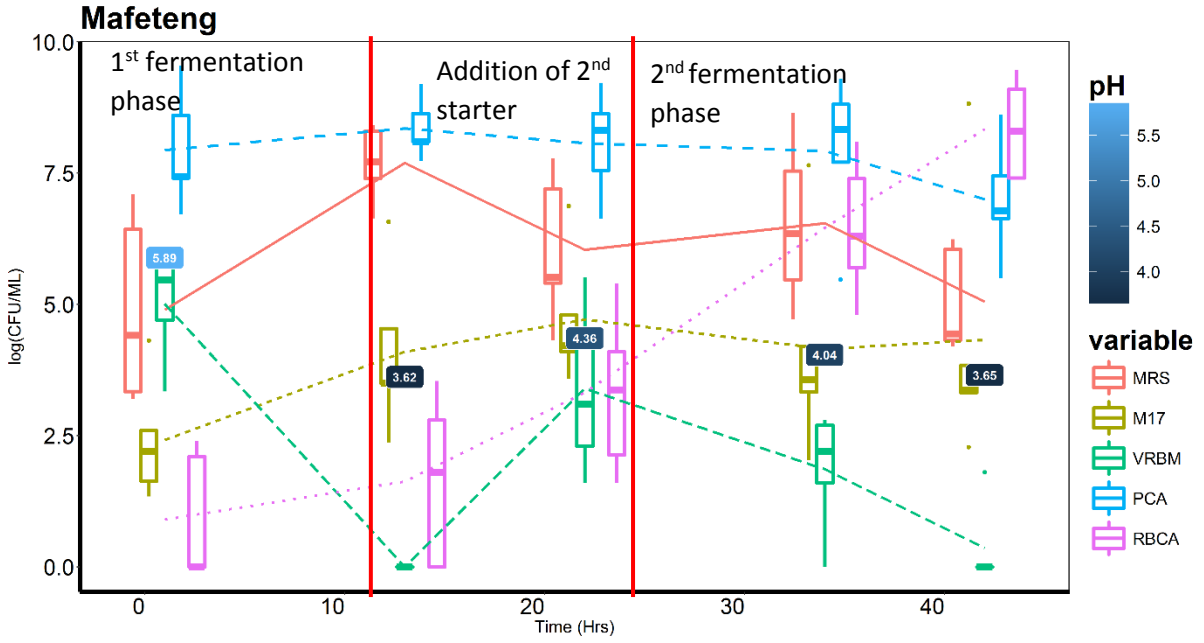

Figure 3B: Microbial patterns of *sesotho* during the fermentation processes in Mafeteng.

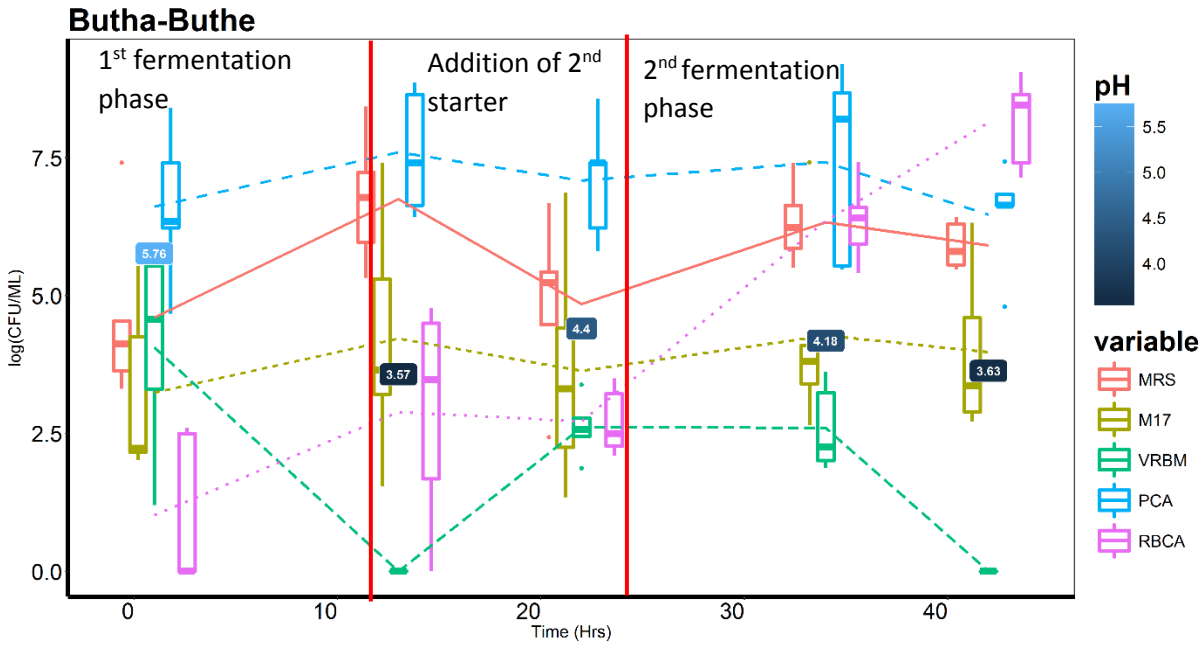

Figure 3C: Microbial patterns of *sesotho* during the fermentation processes in Butha-Buthe.

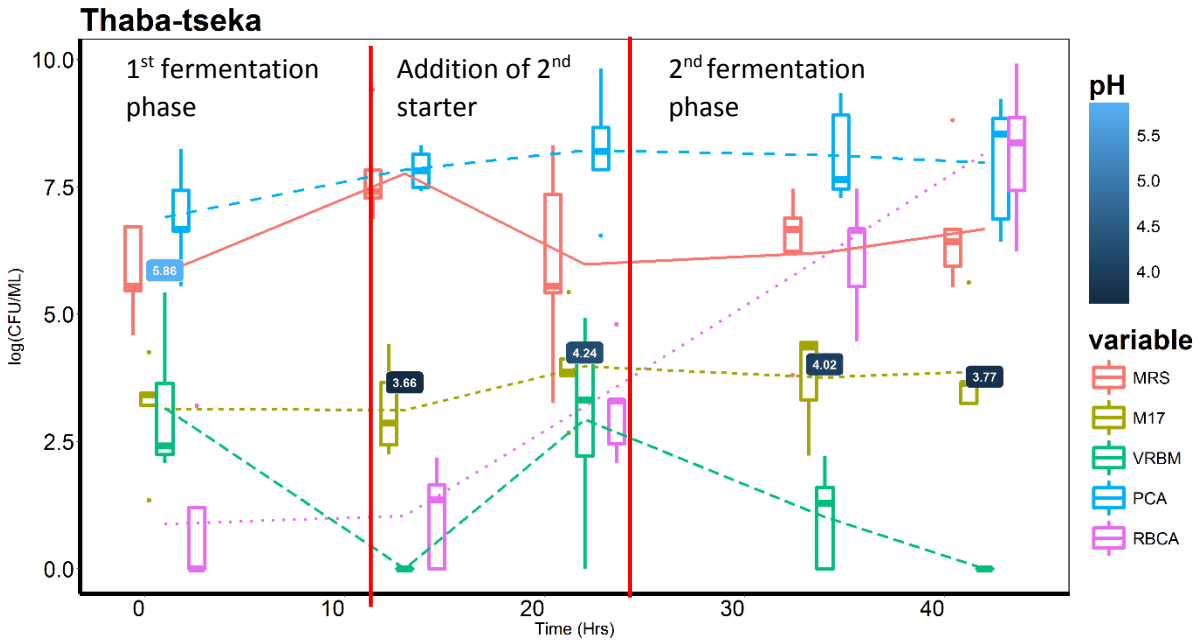

Figure 3D: Microbial patterns of *sesotho* during the fermentation processes in Thaba-Tseka.

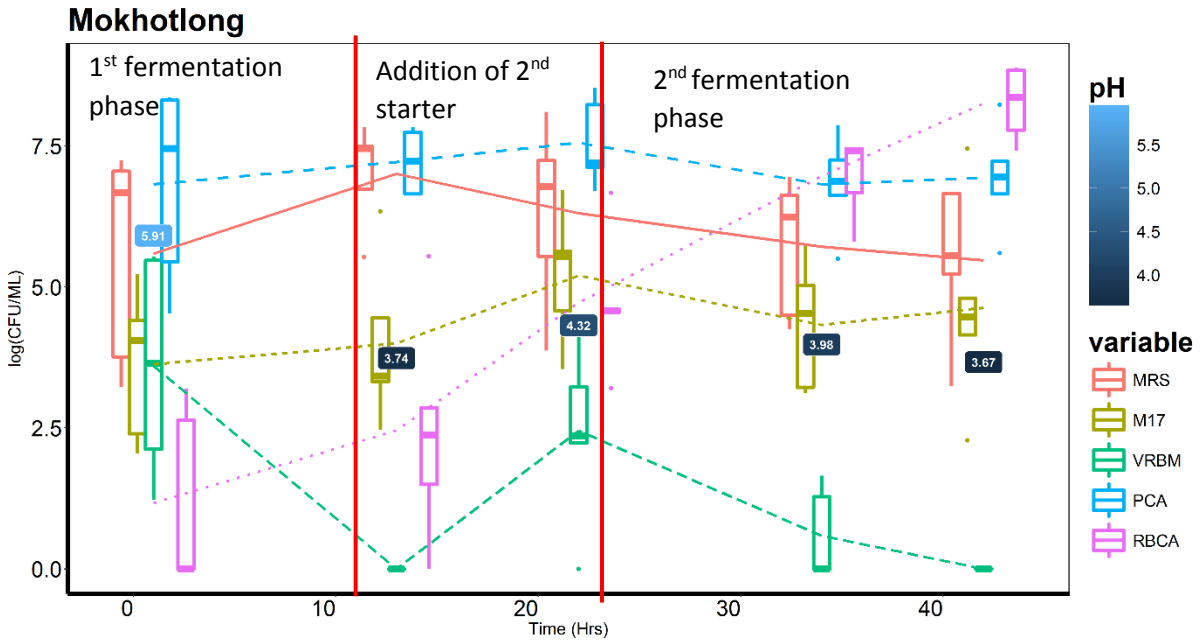

Figure 3E: Microbial patterns of *sesotho* during the fermentation processes in Mokhotlong.

Chapter 4

If the lines for some categories do not extend all the way to the right end of the X-axis, which means that at least one of the samples in that category does not have that many sequences.

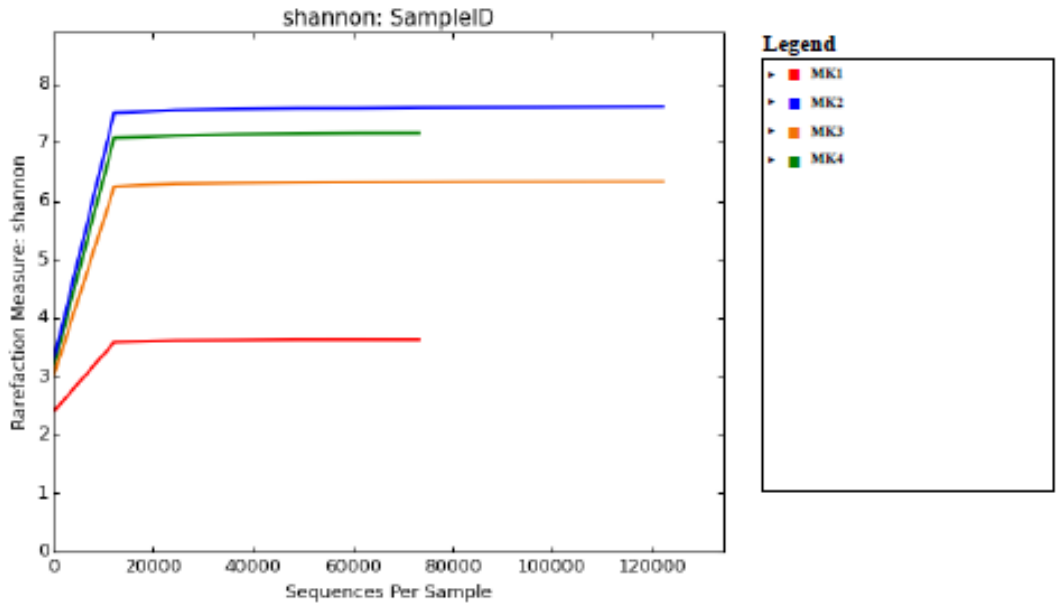

Figure 4A: Rarefaction plot indicating the sequence coverage for Mokhotlong samples.

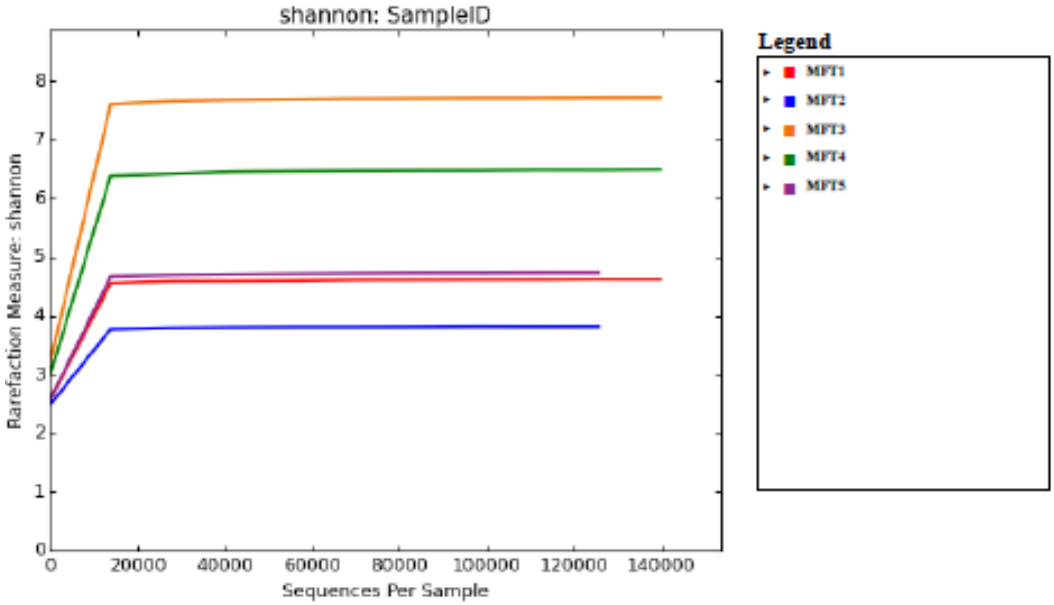

Figure 4B: Rarefaction plot indicating the sequence coverage for Mafeteng samples.

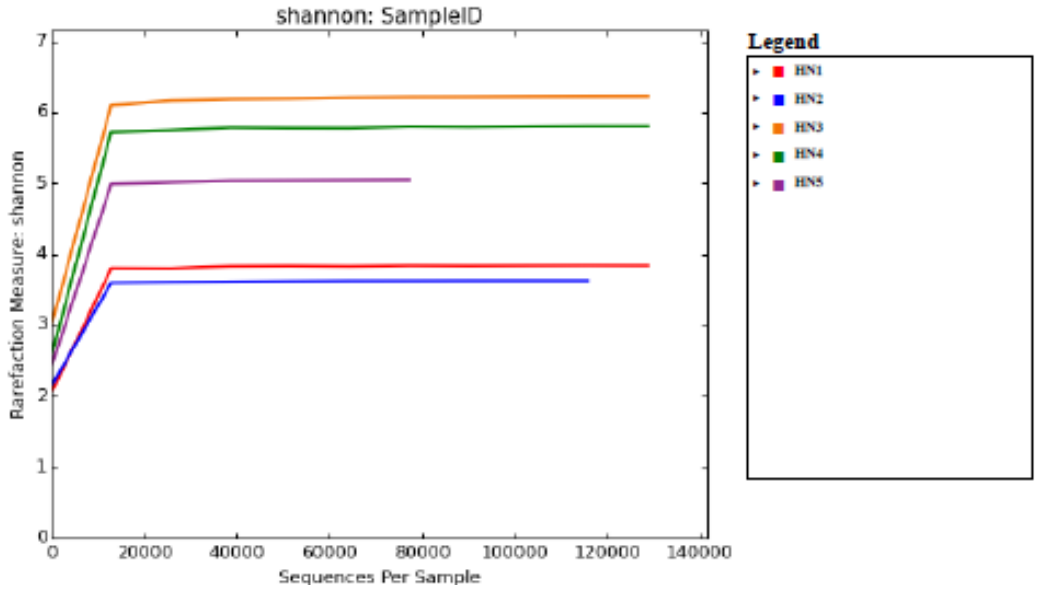

Figure 4C: Rarefaction plot indicating the sequence coverage for Thaba-Tseka samples.

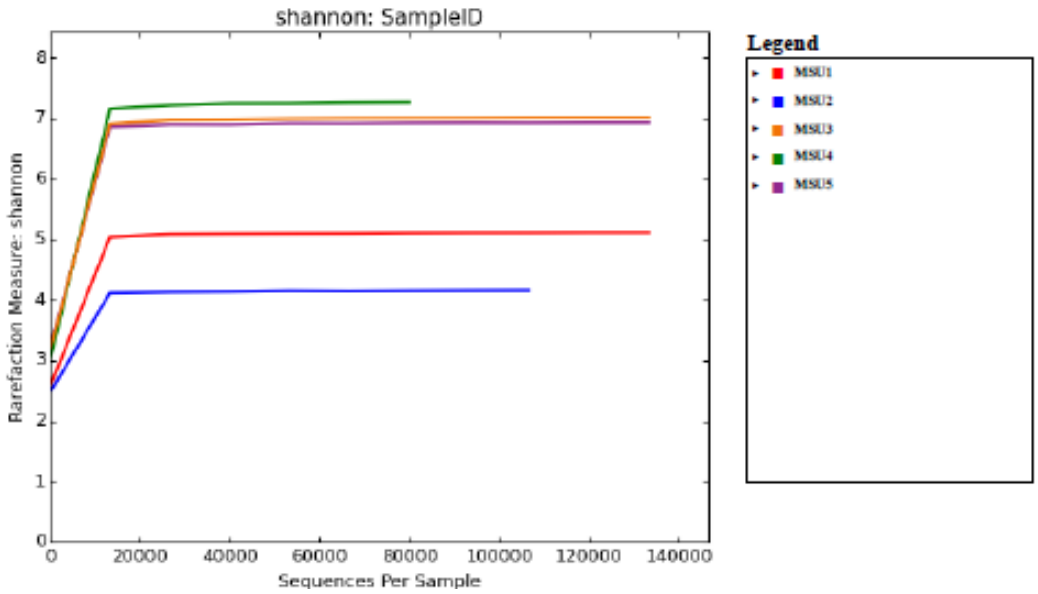

Figure 4D: Rarefaction plot indicating the sequence coverage for Maseru samples.

Table 4E: Geographical information of sites for sample collection.

Location	Latitude	Longitude
Maseru	29.29°S	27.48°E
Mafeteng	29.81°S	27.53°E
Thaba-Tseka	29.52°S	28.64°E
Butha-Buthe	28.82°S	28.51°E
Mokhotlong	29.28°S	29.13°E

Legends of the beta-diversity

Legends continued…

Legends continued…