

**Microbiological and physico-chemical characterization of *khadi*, a traditional
non-cereal based alcoholic beverage of Botswana**



By

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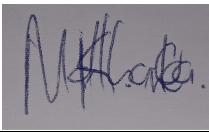
Department of Biological Sciences and Biotechnology

Faculty of Science

Botswana International University of Science and Technology

A Dissertation/Thesis Submitted to the Faculty of Science in Partial Fulfilment of the
Requirements for the Award of the Degree of Master of Science in Biological Sciences and
Biotechnology at BIUST

Supervisor(s): Dr Kebaneilwe Lebani and Dr Nerve Zhou

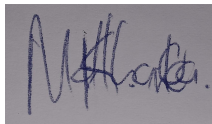
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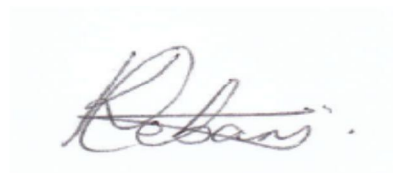
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Dr. Kebaneilwe Lebani

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Abstract

Khadi is a traditional fermented alcoholic beverage popular in Botswana. There is little documented information about the brewing processes, the microorganisms responsible for fermentation as well as the safety despite its popularity in local markets. Such information is vital for production of a standardized beverage with a consistent quality to aid its entrance into the modern commercial markets. This thesis explored the brewing processes, microbiological quality and safety, as important aspects towards a bioprocess development of *khadi*. Firstly, the methods of brewing *khadi* were investigated by administering survey questionnaire to traditional brewers in 1 town and 5 villages in Central and Northern Botswana. The findings suggest that the beverage is produced from the spontaneous fermentation of *Grewia flava* fruits supplemented with brown table sugar. Next, the microbial biodiversity was investigated by isolating microbes from *G. flava* fruits and subsequently genotyping through sequence analysis of D1/D2 domain of the 26S rDNA for yeasts and molds whereas 16S rDNA was used for bacteria. Microbiota responsible for fermentation were further characterized by an *in-silico* restriction fragment length polymorphism of sequenced amplicons (D1/D2 PCR-RFLP). Findings show that fruits predominantly harbored bacteria (77.8 %) with a surprisingly low abundance of yeasts and molds (22.2 %). *Saccharomyces* and non-*Saccharomyces* yeasts dominated the fermented product despite the dominance of bacteria in the fruits before fermentation. Specifically, *Saccharomyces cerevisiae* was the most predominant species (50%) in *khadi* followed *Candida ethanolica* (12.2%) and *Saccharomycodes ludwigii* (10.8%). On average *khadi* contained ethanol concentrations of 5.27 ± 2.02 % (v/v) with an average pH of 3.1 ± 0.31 . Further probing of the identity of the fermenting yeasts using *in-silico* PCR-RFLP, surprisingly, suggested that the *S. cerevisiae* isolates were more genetically identical to the commercial baker's yeast, instead of

either the brewing or wine yeasts as the most common fermentation strains for production of alcoholic beverages. Isolation of spoilage molds such as *Cladosporium sphaerospermum* and *Aspergillus ochraceus* prompted further investigation using the Radox Evidence Investigator and Myco-10 biochip to ascertain the presence of mycotoxins that could render the beverage unsafe for consumption. A variety of mycotoxins were detected although at concentrations below the acceptable limits. Of notable concern, was deoxynivalenol (11.10 ± 5.82 ppb) and diacetoxyscirpenol (22.94 ± 8.15 ppb) with their concentrations being higher than the acceptable limits for a beverage to be deemed safe for consumption. This work is the first to expand the knowledge of the microbial and physiochemical properties of a popular beverage, *khadi*. In addition, this work highlights that a consortium of yeasts is responsible for fermentation of *khadi*. Such findings are critical for recommendation of potential starter cultures for possible commercialization of *khadi* with a consistent quality from standardized processes. The work further characterizes the brewing attributes of yeast isolates such as the ability to utilize different carbon sources, and tolerance to brewing-associated stresses such as high temperature, low pH and high ethanol concentrations. Overall, the results from this work provide perspectives on the strategies for production and improvement of the overall quality and safety of *khadi*.

Acknowledgements

First, I would like to express my sincere gratitude to Botswana International University of Science and Technology for funding my studies. Secondly, my supervisor's **Dr Kebaneilwe Lebani** and **Dr Nerve Zhou** for their continuous support, patience, motivation, enthusiasm, and immense knowledge. Their supervision was of utmost importance throughout the time of my research, including writing of this thesis. I could not have imagined having better advisors and mentors for my study. I would like to thank **Monamodi Kesamang** (a friend and a colleague) for helping me with molecular based techniques on this project, **Kesego Brooks** (MSc student and a friend) for helping me with sample preparations, **Eric Motsamai** (Technician) for helping with media preparation, **Matthews Mokoba** (Senior Technician) for helping me operate most of the equipment in the laboratory, **Hawa Mukami** (MSc student) for her assistance in the chemistry analysis and **Gakeonyatse Modirwagale** (Technician) for her assistance during sample collection. My sincere thanks also go to the rest of the technical staff of the Department of Biological Sciences and Biotechnology for the help they gave during the course of my studies. I am also grateful to my other colleagues for their advice and the stimulating discussions we had.

I would like to extend my gratitude and appreciation to the National Food Technology Research Centre (NFTRC) for allowing me to run some of my experiments in their laboratory.

Above all, I would like to thank God for the success of this project.

Dedication

I would like to dedicate this thesis to my daughter Gofiwa Segwati and my family who stood by me every day and made me who I am today.

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Chapter 1: Introduction

1.1 Introduction

Fermentation is accentuated by the wide spectrum of foods marketed both in developing and industrialized countries. Its benefits to foods range from preservation, safety to improvement of sensory characteristics (Holzapfel, 2002). Traditionally fermented foods are cherished for their contribution to food security, value addition, enhancement of nutritional quality, digestibility and improvement of food safety. These foods are traditionally acceptable and accessible as major nutritional constituents in many developing countries (Holzapfel, 2002; Rolle and Satin, 2002). Preservation and safeguarding of foods and beverages remain the principal objectives of fermentation, with wholesomeness, acceptability and overall quality, having become increasingly valued features to consumers, especially in rural areas where old traditions and cultural particularities in food fermentations are generally well maintained (Marshall and Mejia, 2011).

A large variety of fermented non-cereal-based alcoholic beverages are consumed worldwide. Most fermented alcoholic beverages that are produced commercially in large quantities are from cereals. For instance, in Botswana, *chibuku*, produced from sorghum, has been commercialized (Motlhanka et al., 2018). Standardized and well-controlled production processes have been developed. However, fermented beverages from non-cereal-based substrates in Botswana and beyond are produced from spontaneous fermentation whereby the desirable microbes are naturally present in the raw material. Spontaneous fermentation is unpredictable due to the presence of undesirable microorganisms on the raw materials or fermentation equipment and vessels. Henceforth, there is a need for standardization of processes and development of local food processing capabilities to alleviate food insecurity in Africa (Dendy and Dobraszczyk,

2001). Documentation of traditional food fermentation processes of alcoholic beverages remain a major stumbling block to unlocking the potential of non-cereal-based fermented beverages. Studies on indigenous processes of food fermentation aimed at upgrading or modernizing the methods involved in their preparations are therefore necessary. In Botswana, our fermented non-cereal-based alcoholic beverages include *setopoti*, *bojalwa-jwa-morula*, *muchema* and *khadi*, to list a few (Motlhanka et al., 2018; Sekwati-Monang and Gänzle, 2011).

Khadi is a popular non-cereal-based alcoholic beverage produced from *Grewia flava* fruits in Botswana. However, there is little or no documentation on its production processes and associated safety for consumption despite its popularity and serving as a source of income for many households. Poor documentation and non-standardized production processes could be partly blamed for reported *khadi* poisoning cases recorded in 2010 that led to a ban of *khadi* sales in the Kgatleng region (<https://www.mmegi.bw/index.php?sid=1&aid=159&dir=2010/February/Monday8>) and 2017 (<http://www.themidweeksun.co.bw/?s=khadi>) where five people allegedly died from alcohol poisoning in Ghanzi. Previous work on the characterization of *khadi* only focused on the quantification of the volatile compounds (Mapitse, Okatch, & Moshoeshoe, 2014), leaving leaving the most important factors that affect the overall quality of the product such as a full documentation of the production process, the fermenting microbiota and the safety of the final product uninvestigated. The aim of this work was to document and characterize the brewing process as well as to investigate the microbiota responsible for fermentation of *khadi*. Such information is crucial to ensure batch-to-batch consistency in quality and safety of the beverage. Standardized methods for a safe and hygienic production of *khadi* is of utmost importance for entrance into the commercial markets.

1.2 Rationale of the study

The main aim of this study is to elucidate the microorganisms that are involved *khadi* fermentation processes. Currently, fermentation is carried out under non-standard conditions due to absence of documentation of the processes. Such information is relevant for consistency in microbiological and/or chemical characteristics of the alcoholic beverage. It is noteworthy that discrepancies in processing affect taste, primary and secondary metabolites, result in spoilage and even affect human health.

1.3 Hypothesis and study objectives

1.3.1 Hypothesis

Khadi is a product of spontaneous fermentation of fruits by diverse microbiota and therefore the overall aim of this study was to better understand this fermentation process to improve the fermentation protocol and ultimately the safety of *khadi*.

1.3.2 Objectives

- To document the production process of *khadi* in Botswana.
- To determine the physico-chemical properties of *khadi*.
- To determine the fermentative microbiota on the fruits and *khadi*.
- To evaluate yeast isolates from *G. flava* fruits and *khadi* as potential starter cultures.
- To investigate fungal mycotoxins as a measure of safety for consumption.

Chapter 2: Literature Review

2.1 Fermentation: an overview

Fermentation involves the biochemical modification of foods and food products brought about by enzymes secreted by microorganisms. Fermentation is an energy-yielding catabolic pathway that proceeds with no net change in the oxidative state of the products compared to that of the substrate. This process basically entails the extraction of energy from carbohydrates and other organic substrates without using oxygen as an electron acceptor. Exceptions exist with Crabtree positive yeasts, which tend to ferment even in the presence of excess oxygen and a fermentable carbon source (De Deken, 1966). In the presence of excess sugar and oxygen, a few microorganisms, for example, some yeasts from the *Saccharomycetaceae* family, generate ATP through a respiratory or fermentative metabolism or both pathways concurrently, thus known as respiro-fermentative yeasts (De Deken, 1966).

Crabtree positive yeasts undertake an alternatively exclusive pyruvate dissimilatory route for redox balancing purposes involving the conversion of pyruvate to ethanol. The process recycles NADH by regeneration of NAD^+ essential for ATP production during glycolysis (Navarrete et al., 2014). In comparison to the respiration metabolic strategy, no additional ATP is formed, but fermentation forms a faster oxygen independent ATP production alternative, which is of utmost importance in the utilization of anaerobic niches (Hagman et al., 2013; Piškur et al., 2006; Pronk et al., 1996). In general the absence of a final electron receptor, oxygen, fermentation becomes the cell's primary means of ATP production (Ban and Yang, 1998). A series of glycolytic steps have long been elucidated and their mechanisms well described as shown in Figure 1. In most microorganisms, in the presence of O_2 , NADH and pyruvate are used to generate ATP through the respiratory pathway involving the electron transport chain. This is referred to as oxidative

phosphorylation, and it generates much more energy than glycolysis would alone. As such, cells benefit from avoiding fermentation when oxygen is available with the exception being obligate anaerobes which cannot tolerate oxygen.

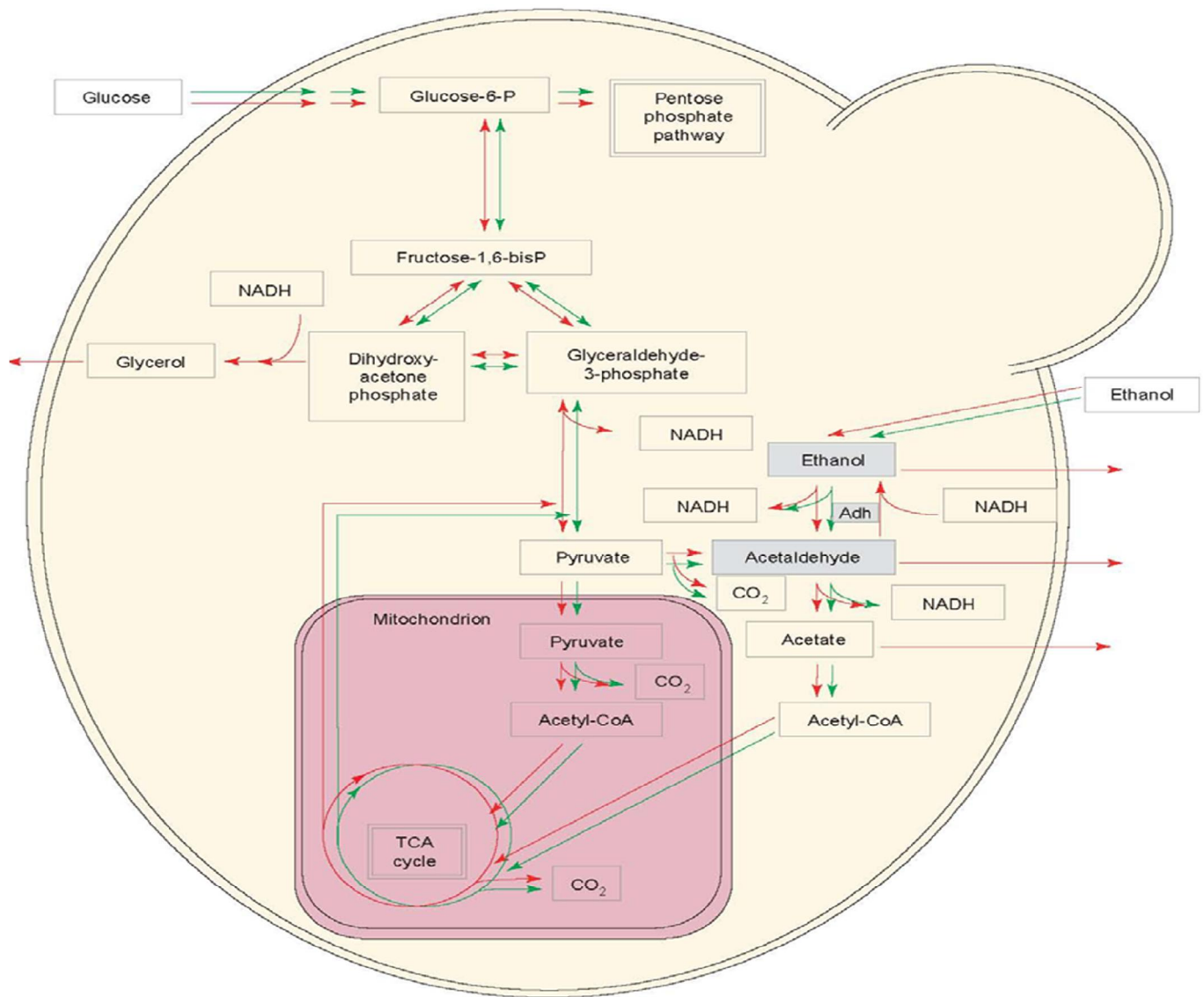


Figure 1: The yeast alcoholic fermentation pathway showing pathways involved in glucose and ethanol assimilation under aerobic conditions (Patrascu et al., 2009).

Glucose is broken down in a series of steps illustrated above (Figure 1) to produce ethanol and CO₂. Based on the fate of pyruvate, at the dissimilatory pyruvate branching point dependent on the oxygen tension and metabolic regulation (Gancedo, 1998; Pronk et al., 1996). Glucose is broken down into pyruvate in cellular cytoplasm in a series of steps, a process called glycolysis. The whole pathway of glycolysis, contains 10 steps of chemical reactions each catalyzed by enzymes namely hexokinase, phosphoglucose isomerase, phosphofructokinase, aldolase, triosephosphate isomerase, glyceraldehyde 3 phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase (Pelicano et al., 2006). In glycolysis, one molecule of glucose (a six-carbon compound) is converted to fructose-1,6-bisphosphate (also a six-carbon compound), which eventually gives rise to two molecules of pyruvate (a three-carbon compound) as seen in Figure 1. Depending on the presence of oxygen, the pyruvate can be broken down either aerobically or anaerobically. In aerobic metabolism, pyruvate loses carbon dioxide and the remaining two carbon atoms become linked to coenzyme A as an acetyl group to form acetyl-CoA, which then enters the citric acid cycle (Pronk et al., 1996). There are two fates for pyruvate in anaerobic metabolism; ethanol production or lactic acid production. In organisms capable of alcoholic fermentation such as yeasts, pyruvate loses carbon dioxide, this time producing acetaldehyde, which, in turn, is reduced to produce ethanol (Jansen et al., 2017).

2.2 Fermentation substrates and fermenting yeasts

Fermentation substrates are the sources of carbon and sometimes they are the source of the fermentative microbes responsible for the fermentation process. One of the most well studied substrates in the production of alcoholic beverages are grapes. Several studies have reported that

grape skins harbor a number of fermenting yeasts. Examples of yeasts isolated from grapes are as follows; *Curvibasidium pallidicorallinum*, *Metschnikowia viticola*, *Rhodosporidium babjevae*, *Sporidiobolus pararoseus*, *Filobasidium floriform*, *Kloeckera* spp., *Hanseniaspora* spp., *Candida* spp., *Pichia* spp., *Kluyveromyces* spp., *Metschnikowia* spp., and *Cryptococcus* spp. (Barata et al., 2012; Bouki et al., 2020; Brysch-Herzberg and Seidel, 2015; Ponzzes-Gomes et al., 2014; Walker and Stewart, 2016). *Hanseniaspora uvarum*, *Pichia terricola*, and *Metschnikowia pulcherrima* have been reported to be the most representative species isolated from grapes (Drumonde-Neves et al., 2017). However, the most well-known fermenting yeast, *S. cerevisiae*, is found in very low frequencies on some grape skins, and in vineyards soils (Swiegers et al., 2009). Some of the non-conventional yeast strains isolated from fermented foods and alcoholic beverages elsewhere in the world include *Brettanomyces* spp., *Candida* spp., *Cryptococcus* spp., *Debaryomyces* spp., *Dekkera* spp., *Galactomyces* spp., *Geotrichum* spp., *Hansenula* spp., *Hanseniaspora* spp., *Hyphopichia* spp., *Issatchenkia* spp., *Kazachstania* spp., *Kluyveromyces* spp., *Metschnikowia* spp., *Pichia* spp., *Trichosporon* spp., *Rhodotorula* spp., *Rhodosporidium* spp., *Saccharomyces* spp., *Saccharomycopsis* spp., *Schizosaccharomyces* spp., *Saccharomycodes* spp., *Sporobolomyces* spp., *Torulopsis* spp., *Torulaspora* spp., *Zygosaccharomyces* spp., and *Yarrowia* spp (Bouki et al., 2020; Tamang et al., 2016a). Non-conventional yeasts are a source of novel traits imparting unique flavors (Capece et al., 2018; Dashko et al., 2015a; Ravasio et al., 2018) in both commercialized and traditional alcoholic beverages like *khadi*. Table 1 summarizes the fermentative microbes isolated from different substrates used to produce non-cereal-based alcoholic beverages.

Table 1: The fermentative microbes from substrates of non-cereal-based alcoholic beverages.

Substrate	Beverage	Country	Microbes	References
Honey	<i>Tej</i>	Ethiopia	<i>Saccharomyces</i> spp., <i>Rhodotorula</i> spp., <i>Debaryomyces</i> spp., <i>Hansenula</i> spp., <i>Lipomyces</i> spp., <i>Oosporidium</i> spp., <i>Pichia</i> spp., <i>Torulopsis</i> spp., <i>Trichosporon</i> spp., <i>Nematospora</i> spp., <i>Schizosaccharomyces</i> spp., <i>Schwanniomyces</i> spp., <i>Torula</i> spp., and <i>Zygosaccharomyces</i> spp.	(Pereira et al., 2016; Snowdon and Cliver, 1996)
Masau (Ziziphus mauritiana)	<i>Kachasu</i>	Zimbabwe	<i>S. cerevisiae</i> , <i>Issatchenkia orientalis</i> , <i>P. fabianii</i> and <i>Aureobasidium pullulans</i>	(Nyanga et al., 2013a, 2008, 2007)
Marula (Sclerocarya birrea)	<i>Mukumbi</i>	Zimbabwe	<i>Aureobasidium pullulans</i> , <i>Geotrichum capitatum</i> , <i>Trichosporon brassicae</i> , <i>Rhodotorula mucilaginosa</i> , <i>H. anomala</i> , <i>H. jadinii</i> and <i>Hansenula</i> spp.	(Okagbue and Siwela, 2002)
Palm sap (Palmae family)	Palm wine		<i>Leuconostoc mesenteroides</i> , <i>L. delbrueckii</i> , <i>Zymomonas</i> spp., <i>Gluconobacter</i> spp., <i>Acetobacter</i> spp., <i>S. cerevisiae</i> and <i>S. chevalieris</i>	(Manel et al., 2011; Santiago-Urbina and Rui'z-Tera'n, 2014; Tapsoba et al., 2015)
Grapes	Wine	China, France, USA, Italy, England, India, etc	<i>S. cerevisiae</i> , <i>Rhodotorula mucilaginosa</i> , <i>P. kudriavzevii</i> , <i>C. parapsilosis</i> , <i>Meyerozyma guilliermondii</i> , <i>Wickerhamomyces anomalus</i> , <i>Kloeckera apis</i> , <i>P. manshurica</i> , <i>C. orthopsilosis</i> , <i>C. zemplinina</i> , <i>H. uvarum</i> , <i>Cryptococcus flaveszens</i> , <i>P. fermentans</i> , <i>C. zemplinina</i> , <i>Cryptococcus carnescens</i> , <i>C. inconpicua</i> , <i>Z. fermentati</i> , <i>Issatchenkia terricola</i> , <i>C. quercitrusa</i> , <i>Hanseniaspora guilliermondii</i> , <i>C. bombi</i> , <i>Z. bailii</i> , <i>Sporidiobolus pararoseus</i> , <i>Cryptococcus magnus</i> , <i>Metschnikowia pulcherrima</i> , <i>Issatchenkia orientalis</i> and <i>P. guilliermondii</i> , <i>H. uvarum</i> and <i>C. flavescen</i>	(Bouki et al., 2020; Chen et al., 2020; Gao et al., 2019; König et al., 2017; Li et al., 2010; Ponzzes-Gomes et al., 2014)

The fermentation of alcoholic beverages in Africa relies on cereals and non-cereal-based substrates. The non-cereal-based substrates include sugarcane (*Saccharum officinarum*), *Berchemia discolor*, palm tree fruits (*Arecaceae* family) and exudates, banana pulp (*Musa acuminata*), watermelons (*Citrullus lanatus*), *Hyphaene petersiana* (*mokolwane/moxao*), *marula* fruit (*Sclerocarya birrea*), *Grewia flava* (*mogwana*), *Grewia occidentalis* (*moretlwa*), *Grewia flavascens* (*mokgomphatha*), *Popowia obovata*, *Balanites aegyptiaca*, *Ziziphus mauritiana* (*masau*), *Kedrostis hirtella* (*mogakangwaga*), *Khadia acutipetala*, cassava (*Manihot esculenta*), and honey (Gadaga et al., 1999; Kubo et al., 2014; Motlhanka et al., 2018; Nyanga et al., 2007; Van Wyk, B.E. and Gericke, 2000) while the cereal-based substrates include maize (*Zea mays*), millet (*Panicum miliaceum*, *Eleusine coracana* and *Pennisetum glaucum*), sorghum (*Sorghum bicolor*), barley (*Hordeum vulgare*), *teff* (*Eragrostis tef*) and wheat (*Triticum aestivum*).

2.3 Cereal-based and non-cereal-based alcoholic beverages

The abundance of a variety of cereals across the African continent could be responsible for popularity of cereal-based alcoholic beverages as compared to non-cereal-based alcoholic beverages. The variety of cereals means that a lot of alcoholic beverages can be produced using these cereals either individually or as mixture. These cereal-based alcoholic beverages are popularly produced using maize, millet, sorghum, barley, *teff* and wheat. The utilization of these different cereals gives the beverages their distinct flavor and taste. The production of cereal-based alcoholic beverages is characterized by malting or germination process prior to fermentation. The common malted beer in Africa are known with different names, for example, *bushera* or *muramba* in Uganda, *chibuku* in Zimbabwe, *dolo*, *burukutu*, and *pito* in West Africa and *ikigage* in Rwanda (Iwuoha and Eke, 1996; Lyumugabe et al., 2010; Muyanja et al., 2003;

Togo et al., 2002). The fermentation process of cereal-based alcoholic beverages is characterized by a two-stage process namely lactic acid fermentation followed by alcoholic fermentation usually carried out by lactic acid bacteria (LAB) and yeast species, respectively (Lyumugabe et al., 2012). The symbiotic relationship between LAB and yeasts is reported to be responsible for production of different secondary metabolites hence distinct aroma complexities among the cereal-based alcoholic beverages (Tamang et al., 2016b). Table 2 below summarizes the fermentative microbiota, physico-chemical properties and secondary metabolites of cereal-based alcoholic beverages of sub-Saharan Africa.

Table 2: Microbial diversity and physico-chemical properties of cereal-based alcoholic beverages.

Alcoholic beverage	Substrate	Sensory property Nature	Alcohol content and pH	Fermentative microbes	Secondary metabolites	Country	Reference
Tella	Barley, wheat, millet, sorghum and <i>teff</i>	Sour, cider taste and light yellow to dark brown	2.5 — 5% (v/v) and 4.5 — 4.8	<i>S. cerevisiae</i> , <i>Lactobacillus pastorianum</i> and <i>Acetobacter</i> spp.	Ethanol	Ethiopia	(Lee et al., 2015; Tafere, 2015; Yohannes et al., 2013)
Amgba	Sorghum and maize	Sour and cloudy	4.5 — 7% (v/v) and 2.5 — 6.2	<i>S. cerevisiae</i> , <i>Kluyveromyces marxianus</i> , <i>Cryptococcus albidus</i> , <i>Debaryomyces hansenii</i> and LAB	Methanol, butanol-1, 2-methyl 1-propanol, 2-methyl 1-butanol, 3-methyl 1-butanol, 1-hexanol, cis-3-hexen-1-ol, trans-3-hexen-1-ol, trans-2-hexen-1-ol, 2-phenyl-ethanol, higher alcohols and acetaldehydes	Cameron and Chad	(Darman Roger et al., 2013; Lyumugabe et al., 2012; Maoura et al., 2005)
Doro	Sorghum, maize, millet	Cloudy and light brown	4% (v/v)	<i>S. cerevisiae</i> , <i>L. plantarum</i> , <i>L. delbrueckii</i> , <i>L. lactis</i> and <i>L. raffinolactis</i>	Ethanol	Zimbabwe	(Gadaga et al., 1999; Jespersen, 2003; Lyumugabe et al., 2012; Togo et al., 2002)

Pito	Sorghum	Golden yellow to dark brown in colour and slightly sweet to very sour	0 — 1.8% (v/v) and 3.9 — 5.3	<i>S. cerevisiae</i> , <i>C. tropicalis</i> , <i>Kloeckera apiculata</i> , <i>H. anomala</i> , <i>Torulaspora delbrueckii</i> , <i>Schizosaccharomyces pombe</i> , <i>Kluyveromyces africanus</i> , <i>Lactobacillus</i> spp. and <i>Leuconostoc</i> spp.	Alkanols, phenols Aldehydes, esters, acids and alkanone/ketones	Nigeria, Ghana, and Togo	(Kolawole et al., 2007; Onyenekwe et al., 2016; Sefa-Dedeh et al., 1999; van der Aa Kühle et al., 2001)
Ikigage	Sorghum	Light yellow to dark brown in colour and sour	2.2% (v/v) and 3.9	<i>S. cerevisiae</i> , <i>Issatchenkia orientalis</i> , <i>L. fermentum</i> , <i>L. buchneri</i> and <i>Lactobacillus</i> spp.	Ethanol and organic acids	Rwanda	(Lyumugabe et al., 2012, 2010; Shanel and Matemu, 2015)
Umcombotsi	Sorghum, maize, bulrush millet or finger millet	Light brown with sour taste		<i>Lactobacillus</i> spp., <i>Leuconostoc</i> spp., <i>Pediococcus</i> spp., <i>Enterococcus</i> spp., <i>S. cerevisiae</i> , <i>S. capsularis</i> , <i>C. ethanolica</i> , <i>C. haemuloni</i> , <i>C. sorbophila</i> , <i>Dekkera anomala</i> and <i>D. bruxellensis</i>	Ethanol	Swaziland	(Kumari et al., 2015; Masarirambi et al., 2009; Simatende et al., 2015; Solange et al., 2014)

<i>Tchoukoutou</i>	Sorghum	Sour taste	2 – 3% (v/v) and 3.61 – 3.98	<i>S. cerevisiae</i> , <i>Torulaspora</i> <i>delbrueckii</i> , <i>S. pastorianus</i> , <i>L. divergens</i> <i>L. fermentum</i> , <i>L. fructivorans</i> and <i>Lactobacillus</i> spp.	Ethanol	Benin	(Greppi et al., 2013; Kayodé et al., 2011; Lyumugabe et al., 2012)
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Non-cereal-based beverages are less generally consumed in comparison to the cereal-based beverages in the sub-Saharan region. The discrepancy could be explained by the abundance of a variety of cereals across the continent. The mass production of non-cereal-based alcoholic beverages of Sub-Saharan Africa relies on uncontrolled spontaneous fermentation and exploits inexpensive substrates that are available in the locality. Some of the beverages made from non-cereal substrate include *muchema*, palm wine, *setopoti*, *bojalwa-ja-morula*, *khadi*, *mukumbi*, *urwagwa*, pineapple wine, and *tej*. The fermentation of non-cereal-based beverages, although not all fully characterized, is reported to be carried out by yeasts, LAB and acetic acid bacteria during a spontaneous fermentation production procedure (Ouoba et al., 2012). Mixed culture fermentation, as shown in Table 3, could influence the distinctive flavor quality of these traditional non-cereal-based alcoholic beverages. Table 3 summarizes the microbial diversity and physico-chemical properties of non-cereal-based alcoholic beverages of sub-Saharan Africa region produced from some of the substrates in Table 1 as well as their secondary metabolites.

Table 3: Microbial diversity and physico-chemical properties of non-cereal-based alcoholic beverages.

Alcoholic Beverage	Substrate	Sensory Properties and Nature	Alcohol content (v/v) and pH	Fermentative microbes	Secondary Metabolites	Country	References
Tej	Honey	Sweet, Effervescent, yellow and cloudy	8.94 — 13.16% (v/v) and pH 3.56 — 4.45	<i>S. cerevisiae</i> , <i>Kluyveromyces bulgaricus</i> , <i>Debaromyces phaffi</i> , <i>K. veronae</i> , <i>Zygosaccharomyces rouxii</i> , <i>Hanensula subpeliculosa</i> , <i>S. norbensis</i> , <i>K. vanudenii</i> , <i>Endomycopsis burtonii</i> , <i>Lactobacillus</i> spp., <i>Streptococcus</i> spp., <i>Leuconostoc</i> spp., and <i>Pediococcus</i> spp.	Ethanol, methanol, esters and organic acids	Ethiopia	(Bahiru et al., 2006; Tamang et al., 2016b; Teshome et al., 2017)
Urwagwa	Banana pulp	Pungent, fruity, herbaceous, astringent/acidic taste and Cloudy	7 — 18.1% (v/v) and no pH information	<i>S. cerevisiae</i>	Ethanol, 1-propanol, 2-hexanol, acetic acid, 5-hexanoic acid, benzoic acid, propanoic acid, formic acid, ethyl acetate, butanoic acid, ethyl ester, 1-Butanol 3-methyl-ethyl ester and hexanoic acid ethyl ester	Rwanda, Burundi, Uganda, Tanzania and Kenya	(Shale et al., 2013)
Palm wine	Palm tree sap	Sour, heavy, astringent taste, whitish and milk-white opalescent liquid	1.5 — 7.1% (v/v) and pH 4.0 — 5.5	<i>Staphylococcus</i> spp., <i>Micrococcus</i> spp., <i>Serratia</i> spp., <i>Bacillus</i> spp., <i>Streptococcus</i> spp., <i>S. cerevisiae</i> , <i>Schizosaccharomyces pombe</i> , <i>C. tropicalis</i> , <i>C. krusei</i> , <i>Candida</i> spp.,	Lactic acid, citric acid, tartaric acids, ethanol and acetic acid, esters, carbonyls, higher alcohols, phenols, sulphur compounds, terpenes,	Nigeria, Bukina Faso and Cameroon	(Nwachukwu et al., 2016; Sampson et al., 2007; Santiago-Urbina and Rui'z-Tera'n, 2014; Steinkraus, 2002)

								<i>Pichia</i> spp., <i>S. chevalieri</i> , <i>Zymomonas mobilis</i> , <i>Hanseniaspora guilliermondii</i> , <i>H. uvarum</i> , <i>L. plantarum</i> , <i>Leuconostoc mesenteroides</i> , <i>L. nagelii</i> , <i>L. sucicola</i> , <i>Acetobacter pasteurianus</i> , <i>A. indonesiensis</i> and <i>Gluconobacter</i> spp.	hydrocarbons, acetals, nitrogen compounds and lactone		
<i>Mukumbi</i>	<i>Marula</i> fruits	Thick creamy liquor and yellowish-brown	15% (v/v) and no pH information					<i>S. cerevisiae</i> , <i>P. anomala</i> , <i>P. guilliermondii</i> , <i>C. tropicalis</i> , and <i>C. intermedia</i>	Ethanol	Namibia, (Misihairabgwi and Botswan Cheikhyoussef, 2017; a, Nyanga et al., 2007)	
										Swazilan d, Zimbab we and Zambia	
<i>Pineapple wine</i>	Pineapples	Fruity, sweet, cream/fatty notes and golden yellowish	6.0 — 7.0% (v/v) and pH 3.78					<i>H. guilliermondii</i> , <i>P. anomala</i> , <i>Meyerozyma guilliermondii</i> , <i>H. uvarum</i> , <i>Wickerhamomyces anomalus</i> and <i>H. opuntiae</i>	Acetic acid, ethyl acetate, isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, ethyl 2-methylpropanoate, 3-methyl-1-butyl acetate, ethyl hexanoate, methyl benzoate, 2-phenylethanol, methyl 2-methylheptanoate, ethyl benzoate, diethyl succinate, ethyl	Angola (Ayogu, 1999; Dellacassa et al., 2017; Pino and Queris, 2010; Roda et al., 2017)	

						octanoate, ethyl phenylacetate, 2-phenylethyl acetate, ethyl 9-decenoate and ethyl decanoate			
Kachasu	<i>Masau</i>	Sour, acidic and clear distillate	9 — 41% (v/v)	<i>S. cerevisiae</i> ,	Isoamyl alcohol, isobutanol, Zimbabwe	(Gadaga et al., 1999;			
	fruits			<i>Issatchenkia orientalis</i> , <i>P. fabianii</i> ,	methanol, acetaldehyde, acetone, we	Nyanga et al., 2007)			
	(<i>Ziziphus</i>		and pH	<i>Aureobasidium pullulans</i> , <i>L. agilis</i> , <i>L.</i>	ethyl acetate, and furfurals				
	<i>mauritiana</i>		information	<i>minor</i> ,					
	),			<i>L. confusus</i> , <i>L. fructosus</i> ,					
	<i>Adansonia</i>			<i>L. bifermentans</i> , <i>L. divergens</i> ,					
	<i>digitate</i>			<i>L. fermentum</i> , <i>L. hilgardii</i> and					
	and			<i>Streptococcus</i> spp.					
	<i>Tamarindu</i>								
	<i>s indica</i>								

Mixed culture fermentation has always been practiced inadvertently during spontaneous fermentation of traditional alcoholic beverages. Substrates are one of the possible sources of fermentation microbes thus the reason for mixed culture fermentation of alcoholic beverages. Mixed culture fermentation characterized by a diversity in carbon metabolism pathways among the different microbes in the consortia, is responsible for production of a variety of primary and secondary metabolites that influence the overall aromatic complexity of the alcoholic beverage (Dashko et al., 2014; Lencioni et al., 2016; Walker and Stewart, 2016). The technique of mixed culture fermentation has become common in the wine and beer industries in an effort to improve the distinctive flavors of the alcoholic beverages. For example, the use of *S. cerevisiae* and *T. delbrueckii* lead to the production of beverages with elevated levels of higher alcohols, ethyl acetate and isoamyl acetate, and lower levels of ethyl hexanoate and ethyl octanoate when compared to the monocultures of the two yeasts (Canonico et al., 2017) thus enhancing the taste and flavor. In the wine industry starter cultures are dominated by *Saccharomyces* species, but the need for new tastes to address diverse customer demands have presented great potential for non-*Saccharomyces* species to be used for mixed culture fermentation (Dashko et al., 2015b; Garofalo et al., 2016).

Mixed culture fermentation utilizing species from both conventional (*Saccharomyces* and *Schizosaccharomyces spp*) and the recently documented fermentative non-conventional genera such as *Kazachstania*, *Naumovozyma*, *Nakaseomyces*, *Tetrapisispora*, *Vanderwaltozyma*, *Zygosaccharomyces*, *Zygorhizula*, *Lachancea*, *Kluyveromyces*, *Eremothecium*, *Dekkera* and others (Hagman et al., 2013) could enhance flavor complexities as well as novel traits (Figure 2). Although some of these species are characterized by poor fermentation, their contribution in mixed cultures is becoming clear.

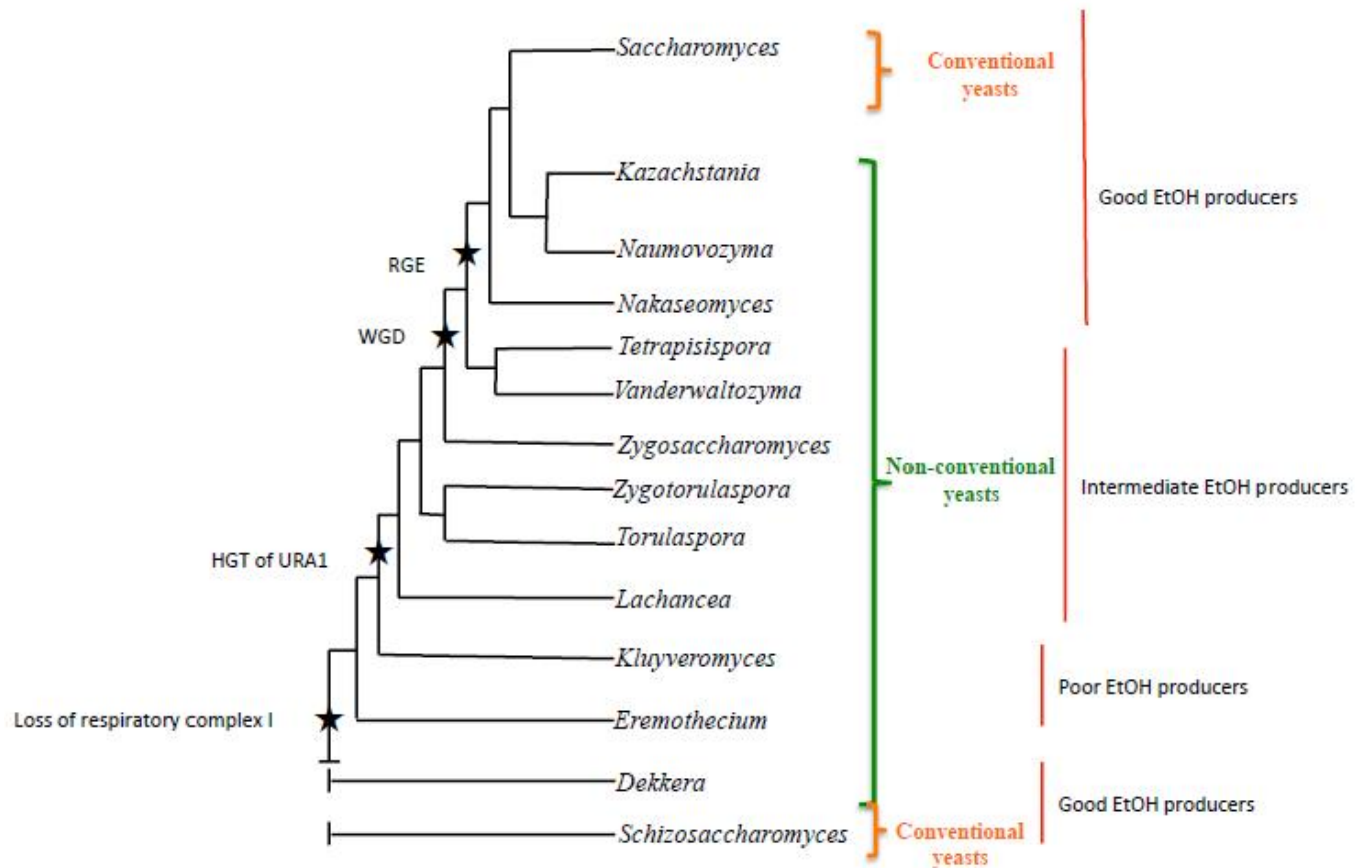


Figure 2: Phylogenetic schematic of conventional and non-conventional yeasts with their fermentation capabilities. This figure was reproduced from (Zhou, 2015). The figure also shows the ethanol production capabilities of respective yeasts.

A consortium in an alcoholic beverage can be made up of yeasts, acetic acid bacteria and (LAB) – which are responsible for the fermentation process of a product. The commonly isolated yeasts include *S. cerevisiae*, *Saccharomyces ludwigii* (Stringini et al., 2009), *C. tropicalis* (Lee et al., 2011), *T. delbrueckii*, *Z. rouxii* (Lleixà et al., 2016), and *P. kluyveri* (Mu et al., 2012). Other than yeasts, some bacteria are known to ferment simple sugars but the end product is usually non-

alcoholic if allowed to ferment exclusively, exceptions being *Zymomonas mobilis*, *Clostridium acetobutylicum*, and *Klebsiella pneumonia* existing (Motlhanka et al., 2018). Major genera of the LAB species isolated from various fermented beverages include *Alkalibacterium* spp., *Carnobacterium* spp., *Enterococcus* spp., *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp., *Oneococcus* spp., *Pediococcus* spp., *Streptococcus* spp., *Tetragenococcus* spp., *Vagococcus* spp., and *Weissella* spp. and these are known for the sour taste and low pH they confer to the product due to lactic acid production.

2.4 Metabolites produced during alcoholic fermentation

2.4.1 Aroma compounds

During alcoholic fermentation, a multitude of primary and secondary metabolites are produced (Styger et al., 2011). Primary metabolites are products of metabolism that are produced during the growth phase of an organism. Primary metabolites perform the physiological functions and support in the overall development of the cell and primary metabolites include hormones, glycerol and enzymes (Dussap and Poughon, 2016; Kong et al., 2007; Navarrete et al., 2014). Secondary metabolites (such as esters, alkanols, organic acids, phenols, aldehydes and ketones), on the other hand, are the end products of primary metabolism that are synthesized after the growth phase has been completed (Figure 3). Such metabolites are vital in the ecological function of the cell. The combination, variety and abundance of aromatic molecules are relevant to sensory properties of alcoholic beverages and thus their unique flavor profile.

Different substrates have different types and amounts of fermentable sugars and amino acids. The presence of different microorganisms in different concentrations during fermentation, each with their varying fermentable sugar preference and primary metabolic requirements leads to

various species-specific pathways being turned on and resulting in production of different secondary metabolites (Tables 2, 3 and 4). These characteristics affect the overall taste and flavor of alcoholic beverages. Differential production of higher alcohols among microbes contributes to the distinctive flavors. Examples of higher alcohols that intensify flavor characteristics of beers include n-propanol, iso-butanol, 2-methylbutanol and 3-methylbutanol (Carrau et al., 2008). Some of the secondary metabolites such as 2-methyl-1-propanol, 2/3-methyl-1-butanol, ethyl lactate and ethyl acetate, which are typical aromatic volatiles reported in beer, wines and spirits, have been isolated in non-cereal-based alcoholic beverages such as *khadi* (Mapitse et al., 2014). Figure 3 summarizes secondary metabolites produced by yeasts.

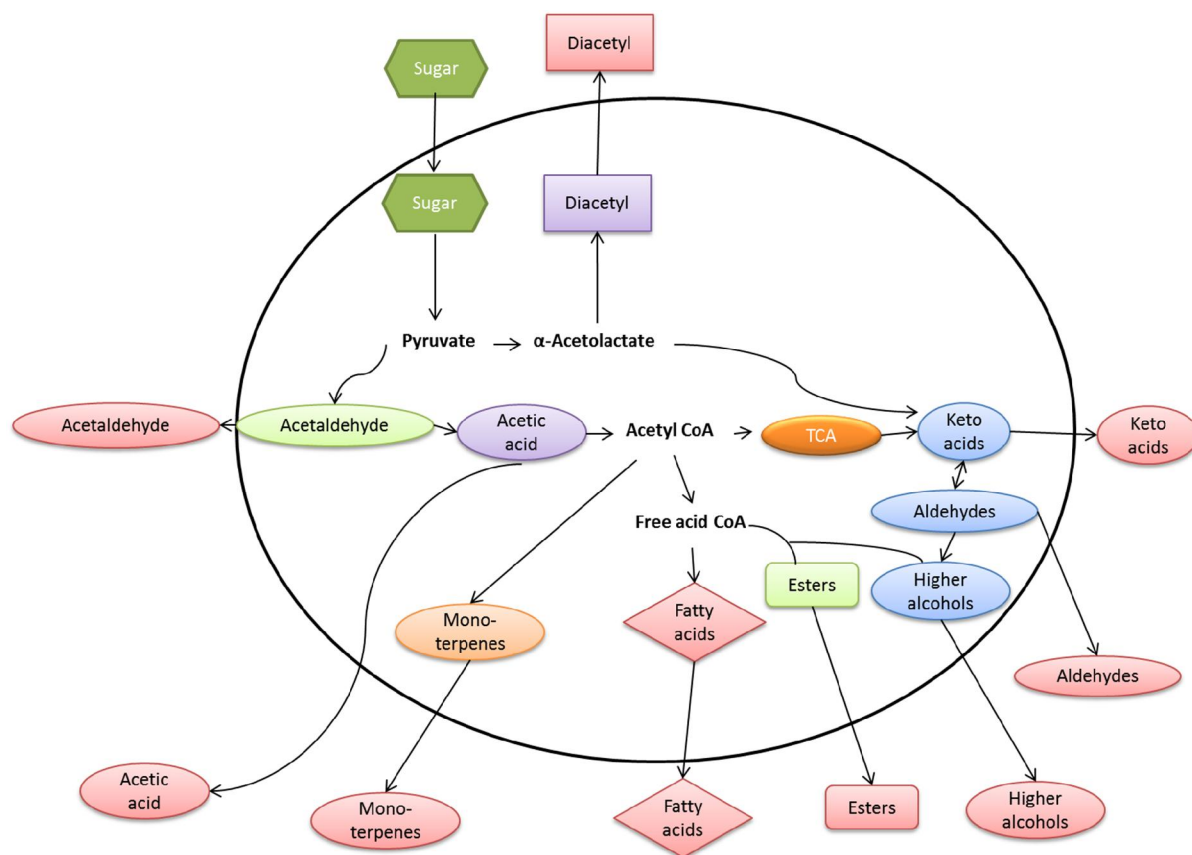


Figure 3: Basic yeast metabolism. Secondary metabolites are shown in (pink) and metabolic intermediates (lime, orange, purple and blue) are also shown (Motlhanka et al., 2018).

Some secondary metabolites act as preservatives, some of which include organic acids such as oxalic, citric, tartaric, malic, ascorbic, fumaric, lactic, and acetic acids. Organic acids are responsible for the rapid acidification of alcoholic beverages (Karamoko et al., 2012). They lower pH to below 4.6, which inhibits the growth of unwanted spoilage microorganisms (Chelule et al., 2010). Not all secondary metabolites contribute positive characteristics to the alcoholic beverages. For example, acetaldehyde has an unpleasant pungent odor at higher concentrations, imparting a freshly cut green leaves flavor to the beverage (Magalhães et al., 2011). Methanol

and its metabolites (such as formaldehyde and formic acid) are produced during fermentation of alcoholic beverages like *kachasu* (Nyanga et al., 2008) and pose health challenges due to their toxicity effects (Arslan et al., 2015). The toxicity of *kachasu* due to the presence of isoamyl alcohol, iso-butanol, acetone, methanol, ethyl-acetate, and furfurals has led to the prohibition of *kachasu* in Zimbabwe since 1971 (Gadaga et al., 1999). Secondary metabolites of alcoholic fermentation are summarized in Table 4 and their contribution to the flavor of the alcoholic beverage is also briefly noted.

Table 4: Contribution of secondary fermentation metabolites to flavor and aroma of alcoholic beverages.

Metabolite class	Examples	Contribution to Flavor and Aroma	References
Organic acids	Succinic acid, acetic acid, lactic acid, citric acid	contribute to the astringency of fermented beverages. The presence of some acids, notably lactic acid, can indicate undesirable bacterial spoilage.	(Manel et al., 2011; Schifferdecker et al., 2014; Swiegers et al., 2009)
Higher alcohols	Isoamyl alcohol, phenylethanol, isopropanol	impart desirable flavor and aromas to fermented beverages but only within certain limits	(Magalhães et al., 2011)
Carbonyl compounds	Acetaldehyde, aldehydes, ketones	Above its flavor threshold in beverages, this compound can impart a “grassy” or “green apple” (related to acetaldehyde) flavor but this can be removed by secondary yeast fermentation during conditioning.	(Hansen et al., 2001; Swiegers et al., 2009)
Vicinal diketones	Diacetyl, pentane-2,3-dione	Diacetyl in most beverages is undesirable, imparting a rancid-butter or “butterscotch” flavor.	(Naydenova et al., 2014; Pereira et al., 2016; Regodón Mateos et al., 2006)
Polyols	Butandiol, propandiol	These compounds make no direct contribution to the aroma, some of them are reactive and can break down with great ease to give pleasant and potent down with great ease to give pleasant and potent volatiles e.g. diendiol.	(Cao et al., 2010; Hansen et al., 2001; Rosa et al., 2017)
Sulfur compounds	Hydrogen sulphide, dimethyl sulphide, sulphur dioxide, Thiols	Dimethyl sulphide (DMS) in low concentration is a desirable attribute of lagers, but higher concentration imparts off-flavors.	(Escudero et al., 2007; Walker and Stewart, 2016)
Esters	Ethyl acetate, ethyl caprylate, ethyl butyrate, ethyl hexanoate, 2-phenethyl acetate, ethyl laurate, ethyl decanoate, hexyl acetate	Associated with a fruity aroma and floral flavors and aromas to fermented beverages.	(De Deken, 1966; Zhou et al., 2017c)

2.4.2 Mycotoxins

Growth of molds on foods and beverages is usually followed by the production of mycotoxins. These secondary metabolites, which are toxic to humans and animals even in small concentrations has been extensively reviewed (Nguegwouo et al., 2018; Roseanu et al., 2010). Mycotoxins production is dependent on many factors such as conditions of storage, ecological and environmental conditions. Poor storage of commodities leads to molds growth which can subsequently lead to spoilage and mycotoxins production (Gori, 2012; Zachariášová et al., 2010). The introduction of mycotoxins in alcoholic beverages could likely be attributed to contamination of raw materials used in the brewing process (Nkwe et al., 2005) and favourable environmental factors such as water, temperature, light, nutrients, and pH (Adekoya et al., 2018b; Sokefun et al., 2018; Zhang et al., 2016). These aspects support the nutritive and proliferative aspects of molds. The mycotoxigenic potential due to mold growth is species or strain dependent (Bennett et al., 2003). Although measures to reduce growth of molds are possible, in most cases not all secondary metabolites are toxic.

Mycotoxins are usually produced by mycotoxigenic genera such as *Aspergillus*, *Alternaria*, *Fusarium*, *Stachybotrys* and *Penicillium* (Kharayat and Singh, 2018; Snowdon and Cliver, 1996). The most common mycotoxins are *aflatoxins*, *deoxynivalenol* (DON), *fumonisin*, *ochratoxins*, *citrinin*, *ergot alkaloids*, *patulin* and *zearalenone* (ZEN) which have previously been reported in beers from Africa and Europe (Ezekiel et al., 2015; Fernández-Cruz et al., 2010; Galvis-Sánchez et al., 2008; Inoue et al., 2013; Kirui et al., 2014; Richard, 2007; Zain, 2011). The significance of the presence of mycotoxins in beverages could be used as an indicator for molds growth on the beverage or the raw materials used. The mycotoxins that have been detected from wines include aflatoxins, ochratoxin A, patulin and *Alternaria* toxins (Matei and Kosseva, 2017).

Some previous work have shown that yeast cells are able to absorb and degrade mycotoxins (Jespersen, 2003; Querol and Fleet, 2012) but alcoholic fermentation does not reduce mycotoxins concentration to non-significant levels thus they are present even after the fermentation process (Inoue et al., 2013). This is made possible by the presence of β -(1 $\rightarrow$ 3) and β -(1 $\rightarrow$ 6)-glucans and mannoprotein, which represent about 20–30 % of the cell dry weight. Table 5 summarizes some mycotoxins that are pertinent to the context of fermentation.

Table 5: Common mycotoxins, their source and their effects on mammalian cells.

Toxin	Source	Effect on mammalian cells	References
Aflatoxin	<i>A. flavus</i> , <i>A. parasiticus</i> , <i>A. bombycis</i> , <i>A. ochraceus</i> , <i>A. nomius</i> , and <i>A. pseudotamar</i>	Carcinogenic	(Bircan, 2009; Falegan and Akoja, 2014; Kharayat and Singh, 2018; Kumari et al., 2015; Lyumugabe et al., 2012; Roseanu et al., 2010; Zain, 2011)
Ergot alkaloids	Sclerotia of <i>Claviceps</i> spp.	Gangrene, central nervous system, and gastrointestinal effects	(Kharayat and Singh, 2018; Lepp, 2010; Morcia et al., 2013; Richard, 2007)
Fumonisin	<i>F. verticillioides</i> , <i>F. moniliforme</i> , <i>F. proliferatum</i> , <i>F. nygamai</i> , and <i>Alternaria alternata</i> f. sp. <i>Lycopersici</i>	Carcinogenic and hepatotoxic	(Bertuzzi et al., 2011; Morcia et al., 2013; Richard, 2007; Roseanu et al., 2010; Rubert et al., 2013)
Ochratoxin (OTA)	<i>A. ochraceus</i> , <i>A. alliaceus</i> , <i>A. auricomus</i> , <i>A. carbonarius</i> , <i>A. glaucus</i> , <i>A. melleus</i> , and <i>A. niger</i>	Carcinogenic, nephrotoxic, hepatotoxic and teratogenic	(Barata et al., 2012; Bertuzzi et al., 2011; Fernández-Cruz et al., 2010; Galvis-Sánchez et al., 2008; Karbancıoğlu-Güler and Heperkan, 2008; Roseanu et al., 2010; Rubert et al., 2013)
T-2 toxin	<i>F. sporotrichioides</i> , <i>F. poae</i> , <i>F. equiseti</i> , and <i>F. acuminatum</i>	Cytotoxic and immunosuppressive	(Richard, 2007; Roseanu et al., 2010; Rubert et al., 2013)
Deoxynivalenol	<i>F. graminearum</i> and <i>F. culmorum</i>	Inhibition of protein synthesis	(Kharayat and Singh, 2018; Nathanail et al., 2015; Rahmani et al., 2009; Richard, 2007; Rubert et al., 2013)

<i>Diacetoxyscirpenol</i>	<i>F. poae</i> , <i>F. semitectum</i> , <i>F. verticillioides</i> , <i>F. sporotrichioides</i> , <i>F. acuminatum</i> , <i>F. culmorum</i> , <i>F. crookwellense</i> , <i>F.</i> <i>venenatum</i> , <i>F. sambucinum</i> , <i>F. equiseti</i> , <i>F. graminearum</i> , <i>F.</i> <i>avenaceum</i> , and <i>F. langsethiae</i>	Cytotoxic and immunosuppressive	(Roseanu et al., 2010; Rubert et al., 2013; Zain, 2011)
<i>Zearalenone</i>	<i>F. roseum</i> and <i>F. tricinatum</i>	Estrogenic activity, potential carcinogenic and teratogenic	(Bertuzzi et al., 2011; Ezekiel et al., 2015; Hill, 2015; Kumari et al., 2015; Roseanu et al., 2010; Rubert et al., 2013)

2.5 *Khadi* – a traditional alcoholic beverage of Botswana

Botswana has a variety of unrecorded traditional alcoholic beverages, which are produced using either cereals, or non-cereal-based substrates. The cereal-based alcoholic beverages include *bojalwa-jwa-setswana* (produced from sorghum) and *sekhokho* (sorghum-based) while the non-cereal-based include *khadi* (produced from *G. flava* fruits) and *setopoti* (produced from watermelon juice) (Motlhanka et al., 2018; Rudloff et al., 2012). This project is focused on the *khadi*, an alcoholic beverage of Botswana produced from *G. flava* fruits and the identification of its fermentative microbiota.

2.5.1 *Khadi*

Khadi is a reddish-brown traditional alcoholic beverage made from the fermentation of ripe sun-dried *G. flava* (*mogwana*) fruits (Figure 4). *Khadi* has been previously reported to contain an alcohol content of between 1.66 % and 5.71 % (v/v), with a pH from 2.87 to 3.16. The beverage has been compared favorably to commercial wine (Mapitse et al., 2014). Brown table sugar is added to the fruit juice before fermentation. One would posit that the sugar is required to aid fermentation of the juice or increase alcohol content. However, there is no available literature documenting the sugar content in the *G. flava* fruits. The other fruits used in *khadi* fermentation include *G. occidentalis* (*moretlwa*), *G. flavascens* (*mokgomphatha*), *Kedrostis hirtella* (*mogakangwaga*), and *Khadia acutipetala* (Van Wyk, B.E. and Gericke, 2000). The most well-known technique used in brewing *khadi* is the back-slopping fermentation technique where an already fermented *khadi* beverage is used as a source of starter culture for the brewing. A Global Status Report on Alcohol consumption by the World Health Organization (WHO) reported that

the mixing in of mashed wild pumpkins, wild fruits, wild tuberous roots, can occur in the production of *khadi* thus making *khadi* to be termed a ‘designer beer’ (WHO, 2004).

Unorthodox methods of production of this beverage have been known amongst the producers. Use of marijuana, car battery acid, tobacco, dagga, during preparation are some well-known practices (Rudloff et al., 2012). The reasons why such harmful additives are used are not documented although some villagers claim that the battery acid increases intoxication (Rudloff et al., 2012). *Khadi* is a unique traditional alcoholic beverage with no similarities to any traditional alcoholic beverage. Mapitse et al., 2014 however detected some volatile compounds from *khadi*, which are present in wine. These volatile compounds are 2-methyl-1-propanol, 2/3-methyl-1-butanol, ethyl lactate, and ethyl acetate. In some respect, the aromatic volatile compounds of *khadi* show comparability to wine. Although the beverage is seemingly popular amongst *Batswana*, there is no literature available documenting the microbiological characteristics of *khadi*. The microbiological characterization and consequent chemical profiling of *khadi* will be important to ascertain the resultant variations of the beverage.



Figure 4: Unripe and Ripe sun-dried *Grewia flava* fruits, a *khadi* fermentation substrate (<http://pza.sanbi.org/grewia-flava>).

Chapter 3: Materials and Methods

3.0 Diversity of microbiota from *G. flava* fruits and *khadi*

3.1 Survey on *khadi* brewing process

A questionnaire was administered to the *khadi* brewers in 1 town and 5 villages in Central and Northern Botswana (Appendix A). The questionnaire survey was carried out to document the process of *khadi* fermentation, the ingredients and their quantities, storage of the ingredients, the fermentation conditions, fermentation duration and the factors to determine completion of fermentation. The questionnaire was completed by a total of 18 respondents, 3 from each location where samples were collected as well as 6 respondents who drink *khadi*.

3.2 Sample collection

During the sampling process, 250 g of ripened sun-dried *G. flava* fruits and 500 mL *khadi* were randomly collected from local *khadi* brewers from Botswana. Collection of samples was only done in central and northern towns and villages (Tonota, Palapye, Serowe, Letlhakane, Mmashoro and Maun) (Figure 5) because *khadi* brewers in the southern part of Botswana use tubers instead of *G. flava* fruits for fermentation of *khadi*. A total of 6 batches of ripened sun-dried *G. flava* fruits and 18 *khadi* samples were collected. *G. flava* fruit samples were collected aseptically using sterile zip-lock bags whereas *khadi* samples were collected in sterile 500 mL bottles. Samples were transported to the laboratory at a temperature of 0 – 4 °C in a cooler box with frozen ice packs before analyses. All samples were analyzed at most 6 hours after collection. All the *khadi* samples were collected at a stage when the *khadi* was ready for consumption i.e. after completion of fermentation.



Figure 5: The map of Botswana showing the sampling locations. The map shows the locations in the central and northern parts of Botswana (black dots) from which ripened sun-dried *G. flava* fruits and *khadi* were collected. The green triangles show the number of replicates per location.

3.3 Physico-chemical characterization of *khadi*

3.3.1 Ethanol assays

Khadi samples were centrifuged and the resultant cell-free supernatant was used to determine the ethanol content using an enzymatic ethanol assay kit (K-ETOH 08-18; Megazyme, Ireland according to the manufacturer's instructions. The samples were analyzed in duplicates.

3.3.2 pH measurement

Within 6 hours of collection and storage at 4 °C, *khadi* samples were tested in triplicate to determine their pH. A pH electrode (HANNA HI208, UK) was directly placed into each sample and the results were recorded. The pH meter was first calibrated using a 3-point calibration steps according to manufacturer's instructions, using buffers standards of pH 2, 7 and 9.0 before use.

3.4 Enumeration and isolation of yeasts and bacteria *G. flava* fruits and *khadi*

3.4.1 Abundance of microbiota isolated from *G. flava* fruits

A modified protocol for isolating microorganism fruits was used from Lee et al., (2011). A maximum of 5 fruits of each of the fruit samples were crushed and re-suspended in 2 mL × PBS (phosphate buffered saline). The mixture was then vortexed vigorously for 5 mins and stored on ice. To isolate yeast, 1 mL from each sample was pelleted in a micro-centrifuge for 1 min at 14 000 x g. The pellets were then re-suspended in 200 µL lysozyme solution (10 mg/mL lysozyme; 20 mM Tris-HCl; 2mMEDTA; 1 % Triton X-100 (pH 8)) and maintained at room temperature for 30 minutes to lyse the bacteria. 100 µL of tenfold serially diluted samples (in 1 × PBS) was then spread plated on yeast peptone dextrose agar (YPD) (2 % glucose; 1 % yeast extract; 2 % peptone; and 1.5 % agar, at a pH of 6.2) supplemented with a cocktail of antibiotics (100 µg/mL streptomycin, 100 µg/mL penicillin and 100 µg/mL ampicillin) for selective growth of yeast before enumeration. The plates were incubated at 30 °C for 48 hours to 72 hours. After incubation, colony-forming units (CFU) were counted and recorded. For further studies, representatives of morphologically distinct colonies were picked, observed under a microscope (DE/Axioplan 2; Carl Zeiss, Oberkochen, Germany) and purified by sub-culturing twice on YPD plates. The pure cultures were then cryopreserved at -80 °C in 25 % glycerol. On the other hand,

isolation of bacteria was done by using the remaining 2 mL of the sample. The samples were serially diluted and spread plated on lysogeny broth agar (1 % NaCl; 1 % peptone; 0.5 % yeast extract; and 1.5 % agar at a pH of 7.4) supplemented with 10 µg/mL of cycloheximide to select against yeast and other fungi. After incubation at 37 °C for 24 hours to 72 hours and representative of different colonies were purified and stored as mentioned above. The experiments were done in triplicates.

3.4.2 Abundance of fermentative microbiota isolated from khadi

100 µL aliquots from all *khadi* samples were 10-fold serially diluted in 1 × PBS, kept on ice, and plated out on either selective YPD or LB agar media to enumerate yeast or bacteria, respectively, as in the section above (section 2.3.1). De Man, Rogosa and Sharpe agar (MRS agar) plates (CONDA Laboratory, Spain) supplemented with 10 µg/mL of cycloheximide were used to determine and enumerate lactic acid bacteria. Plates were incubated at 37 °C (modified method by (Wilson, 2012)) at anaerobic conditions (10 % of CO₂) for 72 hours to promote the growth of LAB. Colonies were counted and expressed as log colony-forming units per milliliter (log CFU/mL) and recorded. The experiments were done in triplicates of triplicates meaning each sample was plated onto 27 plates.

3.4.3 Isolation of molds from khadi samples

Isolation of molds was done as in section 3.4.2. The mold colonies on the YPD plates were then cut and transferred to malt extract agar (MEA; 3 % malt extract, 0.5 % mycological peptone, 1.5 % agar and pH 5.4) (Oxoid, England) and potato dextrose agar plates (PDA; 0.4 % potato infusion, 2 % dextrose, 2 % agar and pH 3.5) (Difco, Detroit, USA) to get pure cultures which were then incubated at 30 °C for 5 days. Purified cultures were stored in 25 % glycerol at –80 °C for further analysis.

3.5 Identification and characterization of bacterial isolates from *G. flava* fruits

3.5.1 DNA extraction and PCR from bacterial isolates

DNA was extracted from a single colony using GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) according to manufacturer's instructions. The extracted genomic DNA was used for PCR amplification using 16S rDNA universal primers, pAF (5'-AGAGTTTGATCCTGGCTCAG-3') and pER (5'-CCGTCAATTCCTTTGAGTTT-3') (Ouoba et al., 2004) and the following cycling conditions: initial denaturation at 98 °C for 30 s; 35 cycles of denaturation (98 °C for 30 s), annealing (47 °C for 30 s), and extension (72 °C for 30 s), a final extension step at 72 °C for 7 min and held at 4 °C ∞. Negative controls in which the template DNA in the PCR mixture was replaced with sterile distilled water were also included. All amplicons were purified using a QIAquick PCR product purification kit (Qiagen, GmbH, Germany) according to manufacturer's instructions. The amplicons were sequenced by Inqaba Biotech (Pretoria, South Africa). SnapGene® Viewer software ver. 4.2.11 (GSL Biotech) sequence editing tool was used to generate contiguous sequences (<http://www.snapgene.com>). Species identification was done by using a BLAST nucleotide sequence analysis tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

3.5.2 Characterization of bacteria from *G. flava* fruits

Bacterial isolates were subjected to biochemical tests which included Gram staining, lactose test (LAC), indole test (IND), methyl red test (MR), Voges-Proskauer test (VP), urease test (URE)

and catalase test (CAT). Gram staining was performed by staining a single with crystal violet and counterstaining it again with safranin and observing the Gram type under a microscope. Lactose test was performed by inoculating the lactose broth with the bacterial cultures and the broth was incubated at 37 °C for 24 hours. The positive results were evident when there was a color change from yellow to a pink or red color at the surface of the medium while negative results had no color formation. The IND test was performed by inoculating a loopful of bacterial culture into 2 mL tryptone water and incubating the vials at 37 °C for 24 hours. After incubation, the Kovac's reagent was added to the broth. The positive results were characterized by a pink layer while negative results had no pink layer formation. The MR test was performed by inoculating cultures into methyl red-Voges-Proskauer medium (MR-VP medium) and incubation at 30 °C for 3 days. After 3 days, 5 drops of methyl red solution were added and further incubated for 48 hours at the same temperature as above. The positive results were a red color formation and the negative results were a yellow color. Under the VP test, the MR-VP medium was inoculated with the cultures and incubated at 37 °C for 48 hours. After incubation, 0.6 mL of alpha-naphthol was added to the broth and 0.2 mL of potassium hydroxide. The tubes were then shaken by hand for 1 minute to expose the medium to atmospheric oxygen in order to oxidize the acetoin and allowed to stand for 10 minutes. The positive results were a red color formation while the negative results were no color formation. The URE test was performed by inoculating a loop full of the culture to the urease broth with the cultures and the broth was incubated at 37 °C for 48 hours. The positive results were a color change from yellow-orange to bright-pink and the negative results were no color change. The CAT test was performed by adding a small amount of the bacterial culture into a 100 µL freshly prepared 1 % hydrogen peroxide. The positive results were production of bubbles while the negative results were no bubble production.

3.6 Identification of yeast isolates from *G. flava* fruits

A single colony from each of the yeast isolates with distinct differences in morphology was picked and purified by re-streaking on YPD. These colonies were then re-suspended in 50 μ L sterile deionized water. The mixture was then boiled at 98 °C for 15 mins and then centrifuged for 1 min at 16 000 $\times$ g using a micro-centrifuge. 2 μ L was then used as a template for PCR amplification of a 560 bp DNA fragment of the 26S rDNA using universal PCR primers, NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') as reported elsewhere (Kurtzman and Robnett, 1997). PCR amplification was performed using Applied Biosystems ProFlex PCR systems in a 20 μ L (containing 2.5 μ L 10 $\times$ PCR buffer, 2.0 μ M of each primer, 0.2 mM dNTPs, and 1.25 U *rTaq* DNA polymerase (Takara Bio Inc., Japan) using the following cycling conditions: initial denaturation at 98 °C for 3 min; 38 cycles of denaturation (98 °C for 15 s), annealing (54 °C for 1 min), and extension (72 °C for 1 min), a final extension step at 72 °C for 7 min and held at 4 °C ∞ . Negative controls were as in the above section (section 2.4.1). PCR product purification, sequencing and identification of yeast were done as in the previous section above (section 2.4.1) as well as using a/the pairwise identification tool available on the Westerdijk Fungal Biodiversity Institute (CBS-KNAW) strain database, (<http://www.westerdijkinstitute.nl/>). The control yeast used in this study is a commercial baker's yeast obtained from local supermarkets. It should be noted that there are no retail outlets in Botswana that sell commercial brewer's yeast that could have been used as a control in this study.

3.7 Identification and characterization of yeast isolates from *khadi*

3.7.1 Yeast colony PCR

Yeast colony PCR and identification were carried out as outlined in section 3.6.

3.7.2 Phylogenetic analyses

To determine the phylogenetic relationship of the isolates from the *khadi* samples, we used MEGA 7.0.26 software (Kumar et al., 2016). The sequences were aligned using MUSCLE within the MEGA software and then the aligned sequences were used to calculate the best maximum likelihood model (Jukes-Cantor, Kimura 2-parameter, Tamura 3-parameter, Hasegawa-Kishino-Yano, Tamura-Nei and General time reversible models) to be used to construct the phylogenetic tree.

3.7.3 In-silico PCR-RFLP of *Saccharomyces* and non-*Saccharomyces* yeasts

We sought to investigate all *S. cerevisiae* isolates that showed a significantly high percentage of global similarity to the readily available baker's yeasts to ascertain whether the wine makers used it as starter cultures or depended on the naturally occurring yeasts isolated from the dried fruits. To do so, we performed an *in-silico* PCR-RFLP (restriction fragment length polymorphism) using 26S rDNA amplicons with a SnapGene® Viewer software ver.4.2.11 (GSL Biotech) (<http://www.snapgene.com>). The simulation of gel electrophoresis with a set parameter of 4 % agarose gel option was chosen for visualization after an *in-silico* digestion with the following restriction enzymes *GluI*, *HaeIII*, *HinfI* and *RsaI*. pUC19 – *Sau3AI* digest was selected as a molecular weight ladder to compare the mobility of the fragments.

We also used the same technique to investigate whether the same non-*Saccharomyces* yeasts found among wines from across Botswana were similar. The 26S rDNA sequences of the ale

yeasts (*S. cerevisiae*, SafAle T-58, Fermentis, France), lager yeasts (*S. pastorianus*, Lallemand Brewing, Austria) and wine yeasts (*S. cerevisiae*, Lalvin EC-1118, Lallemand Brewing, Austria) where downloaded from NCBI (<https://www.ncbi.nlm.nih.gov/>). These where ran in parallel with readily available commercial baker's yeast.

3.8 Bioprocess development

To select potential starter culture yeasts to be used for a standardized production of a commercial grade of *khadi*, the yeasts isolated from both the *G. flava* fruits and *khadi* were tested. The tests were aimed at identifying the best yeast isolates with the following attributes; higher fermentative capacity, ability to utilize different carbon sources as well as those yeasts with higher stress tolerance to brewing-associated stresses. Such attributes are important for an effective fermentation process.

3.8.1 Carbon assimilation in yeasts

The utilization of a wide substrate range is one of the important attributes for potential brewing starter cultures. The attributes determine the economical production of beer and determine the attenuation ability responsible for either low alcohol or high alcohol beverage. *G. flava* fruits like any other fruits contain multiple fruit sugars (not determined in this study) and thus a yeast capable of converting these sugars into ethanol will be an ideal starter culture. Therefore, the ability of the yeast isolates to assimilate selected carbon compounds was tested using the API 20C AUX kit (Bio-Mérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. The yeast isolates were also grown in different locally available sugars [maltose (2 %), fructose (2 %), sucrose (10 % and 20 %) and lactose (2 %)] to confirm their utilization of the sugars.

3.8.2 Yeast characterization: Determination of fermentative capacity.

The ability to ferment reducing sugars and other carbon sources found in brewing substrates into ethanol is one indispensable attribute of a brewing yeast starter culture. The isolates were therefore tested for their fermentative capacity as reported by (Zhou et al., 2017b) with minor adjustments. In brief, a single colony of each of the isolated strains and the controls (ale yeast, baker's yeast and wine yeast) were grown overnight in media containing *G. flava* fruits (200 g/L) and brown sugar (GFS media) (100 g/L) in 5 mL test tubes. The samples were incubated in an incubating shaker at 180 rpm at 30 °C. Overnight cultures were pelleted, washed with sterile deionized water and used for inoculation in GFS media. The GFS media (3 mL) was inoculated with a yeast suspension culture to give an initial OD_{600nm} of 1.0 and introduced into a 30 mL marked syringes in triplicates. The syringes were incubated in a shaker at 30 °C at 180 rpm for 24 hours. Fermentative capacity was determined by measuring carbon dioxide evolved during fermentation, which corresponded to the movement of the syringe plunger. The results were recorded at hourly intervals until fermentation was complete. The experiments were done in duplicates and repeated twice.

3.8.3 Yeast stress tolerance

Yeasts are subjected to a variety of stresses during brewing. These stresses impact their fermentative performance, which subsequently affect the overall quality of a beverage. Investigating the capacity of the potential starter cultures to withstand osmotic, ethanol, pH, and high temperature fermentation stresses associated with brewing is essential. Specific isolates from *G. flava* fruits and *khadi* used in stress tolerance tests were grown overnight in liquid YPD in 5 mL tubes at 30 °C and 180 rpm on a shaker as described elsewhere (Zhou et al., 2017b). The

cells were harvested by spinning down at 13 000 rpm for 2 minutes and washed with deionized sterile water. The cells were then adjusted to OD_{600 nm} of 0.5 in phosphate buffered saline. The cultures were further diluted in 96-well plates to give working OD_{600 nm} of 0.5, 0.25 and 0.125. Cells in these dilution ranges were then spotted using a spot test stamp (replicator) on different solid media plates containing YPD supplemented with different reagents to test for different stresses. For osmotolerance, YPD was supplemented with different NaCl concentrations to working concentrations of 0.5M, 1M, 1.5M, 2M and a control with a working concentration of 0M NaCl. To test for thermotolerance, isolates were spotted on YPD plates and incubated at different temperatures (37, 40, 41, 42, 43 and a control temperature of 30 °C). pH stress tolerance was tested by growing the yeasts in liquid media set at different pH (1, 2, 3, 5 and a control pH of 6.2). The ability of isolates to tolerate different ethanol concentrations was also tested by spotting isolates on media with different ethanol concentrations (3, 5, 7, 9 and a control with 0 % ethanol). In all other tests for stress, incubation was done at 30 °C. The ability to tolerate different types of stressors was evaluated semi-quantitatively by estimating the abundance of colonies that grew on a control plate in comparison to those that grew on a selective media plate. The plates were photographed every 24 hours and the plate photos with the best differences were recorded.

3.9 Safety of *khadi*

Isolation of molds prompted further investigation to ascertain the presence of mycotoxigenic fungi. Since, no bacteria isolates or pathogenic yeasts were isolated these microorganisms were not considered for investigation of safety of *khadi*.

3.9.1 DNA extraction and identification of molds isolates

The molds were collected and cultured as described above (Section 3.4.2). Genomic DNA was purified by the microwave method (Bollet et al., 1991). The fungi mat was rinsed with 1 mL TE buffer, centrifuged, and lysed with 100 µL TE buffer and 50 µL 10 % SDS. The molds mat was then incubated at 65 °C for 30 min and centrifuged to remove the supernatant. The cell pellet was placed in a microwave oven, heated twice for 1 min at 900 W. The pellet was then resuspended in 200 µL TE buffer with one volume of phenol: chloroform: isoamyl alcohol (25:24:1) for 15 min. The aqueous phase was recovered by centrifugation; the DNA was precipitated with 95 % ethanol and centrifuged at 12 000 rpm for 20 mins. Then, the DNA was rinsed with 1 mL 70 % ethanol and resuspended in 200 µL deionized water for PCR assays. PCR conditions and identification were carried out as outlined in Section 3.6.

3.9.2 Mycotoxin quantification

3.9.2.1 Radox Evidence Investigator Myco-10 calibration

The Radox Evidence Investigator was calibrated based on the nine-point calibration using Radox Evidence Investigator Myco-10 calibrator, which covered the calibration range of all assays. The calibration data was used to calculate the linearity, LOD and LOQ of the calibration process. The LOD is the detection limit of an analytical procedure is the lowest concentration of analyte in a sample that can be detected while the LOQ is the smallest concentration of a measurand that can be reliably measured by an analytical procedure.

3.9.2.2 Extraction of mycotoxins from khadi samples

After calibration, a total of 2.5 mL of each *khadi* sample was mixed with 20 mL pure Acetonitrile (Sigma–Aldrich, USA) in a 50 mL centrifuge tube. Sodium chloride (2 g) and magnesium sulphate (0.25 g) were added to the mixture. The resultant mixture was vortexed for 3 minutes followed by centrifugation at 4000 rpm for 10 minutes at 4 °C.

3.9.2.3 Mounting of samples onto a Myco-10 biochip

150 µL of diluted wash buffer was pipetted into each of the 9 biochip wells (Myco-10 biochip, Randox Diagnostics, UK). 50 µL of the extracted sample was added to the appropriate biochip well. After gently tapping the edges of the handling tray to mix the samples and reagents, the trays were placed in the thermo-shaker and incubated at 25 °C for 30 minutes at 370 rpm. A working strength solution conjugate of 100 µL was pipetted in each biochip well. The biochips were then incubated at 25 °C for 60 minutes at 370 rpm. The biochips were then immediately washed twice by pouring 375 µL of wash buffer to each biochip well. Another wash cycle was carried 4 times and the biochips were finally soaked in wash buffer for 2 minutes. After the final wash, the biochips were tapped onto a lint free paper towel to remove any residual wash buffer. After tapping, the biochip wells were filled with wash buffer and left to soak until directly prior to imaging.

3.9.2.4 Myco-10 biochip imaging and mycotoxins quantification

Each biochip carrier was imaged individually and biochips awaiting imaging were protected from light by covering them with a lint free paper towel. Each biochip well was tapped onto a lint free paper towel to remove the wash buffer. A signal reagent of 250 µL was added to each biochip well and covered to protect from light for 2 minutes. The biochip carrier was placed into

the Evidence Investigator (Randox Diagnostics, UK), images from each well were captured and mycotoxins per well quantified. In Evidence Investigator, degree of binding is determined using a chemiluminescent light source, quantified using a Charge Coupled Device (CCD) camera and imaging system. The Randox Evidence Investigator detects and quantifies a total of 10 mycotoxins per sample (paxilline, fumonisin, ochratoxin A, aflatoxin G1/G2, ergot alkaloids, diacetoxyscirpenol, deoxynivalenol, aflatoxin B1/B2 and zearalenone). The control provided with the kit of a known concentration was ran along with the samples to monitor the efficiency and accuracy of the kit.

The extraction method was confirmed for accuracy and efficiency by spiking 3 samples from 3 different sampling locations with known concentrations of AFB1 (0.1 ppb), FB1 (1 ppb) and OTA (2 ppb). The Myco-10 extraction method was also used as instructed by the manufacturer (Randox Diagnostics, UK) and compared with the above extraction method. The method involved taking 5 mL of the *khadi* sample and adding 25 mL of acetonitrile: methanol: water (50:40:10). The mixture was then vortexed for 60 seconds and hand rolled for 10 minutes. After rolling, the mixture was centrifuged for 10 minutes at 3 000 rpm to complete the extraction. The mycotoxin quantification and spiking were done as stated above.

3.11 Statistical analyses

One-way ANOVA was used to compare differences among the CFUs/mL of yeasts isolated from the *khadi* samples (Microsoft Office 2019, USA). The baker's yeast was used as a control isolate in the gassing power experiment and the differences in the gassing power between each *khadi* isolate and the control bakers' yeast was analyzed using a t-test.

Chapter 4: Results and Discussion

4.1 *Khadi* brewing process

4.1.1 Survey responses

Khadi brewing process employs a back-slopping technique in which the previously fermented fruits (*seretse*, 30 L) is pitched as the raw material for the next fermentation step. To supplement on the available fermentable sugars in the ripe sun-dried *G. flava* fruits (20 L), table brown sugar at a concentration of 10 kg is added. Then fermentation is allowed to proceed for 18 to 24 hours or 36 to 48 hours depending on the ambient temperature (Figure 6). Once the fermentation time is reached, the product is sieved using old repurposed sacks before consumption. Repitching of the previous fermented *G. flava* fruits was done for a maximum of 2 times. Figure 6 summarises the *khadi* fermentation process.

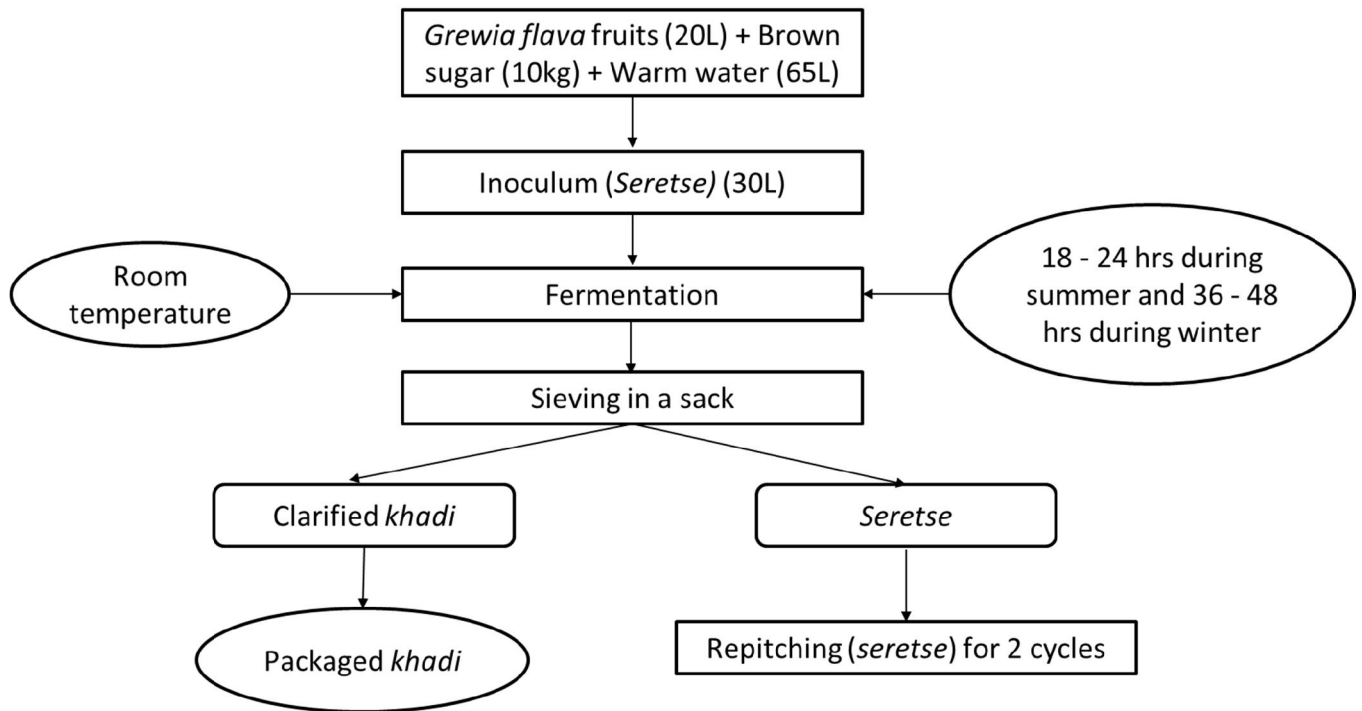


Figure 6: Summary of the *khadi* fermentation process for a fermentation of 100L.

The responses from the questionnaires for *khadi* brewing process were analysed (Figure 7). The results show that *khadi* is mostly produced by people who are not formally employed (80 %) and the minority (20 %) are retired or in formal employment. The trend suggest that most brewers probably depend on it as a source of income. In addition, the results suggests that *khadi* is brewed using the following main ingredients; *seretse* (inoculum), *G. flava* fruits, brown table sugar and warm water (Figure 7b).

The use of wild fruits in traditional beverages has been reported elsewhere for the production of *mukumbi* of Zimbabwe produced from marula fruits (Dlamini and Dube, 2008; Mpofu et al., 2008). Production of *khadi* is similar to *mukumbi* but sugar supplementation is not carried out in the brewing process.

It should be noted that some brewers added grapes although it was observed among a few brewers. In contrast to expectations that the brewers used some unique brewing pots, it was found out that fermentation was done in tightly closed repurposed drums. Another observation is that fermentation was done at room temperature which fluctuates seasonally and thus could be one of the reasons for batch-batch variations. Surprisingly, some drums used in brewing did not have lids but the brewers used large clear clean plastics and blankets to close the drums. Such a setup creates differences in the final product quality as differences in amounts of oxygen in the fermentation vessel affect the metabolism of yeasts which could oscillate between respiration and fermentation. Therefore the amounts ethanol should not be expected to be uniform in such setups.

Khadi brewers determine completion of fermentation in 3 different ways that include taste, bubbles and foam. Completion of fermentation includes aspects such as change in taste from sweet to the *khadi* distinct taste, reduction in bubble production and disappearance of the foam.

Given that *khadi* is produced under non-standardised brewing conditions, it is noteworthy that sugar and alcohol testing are not carried out

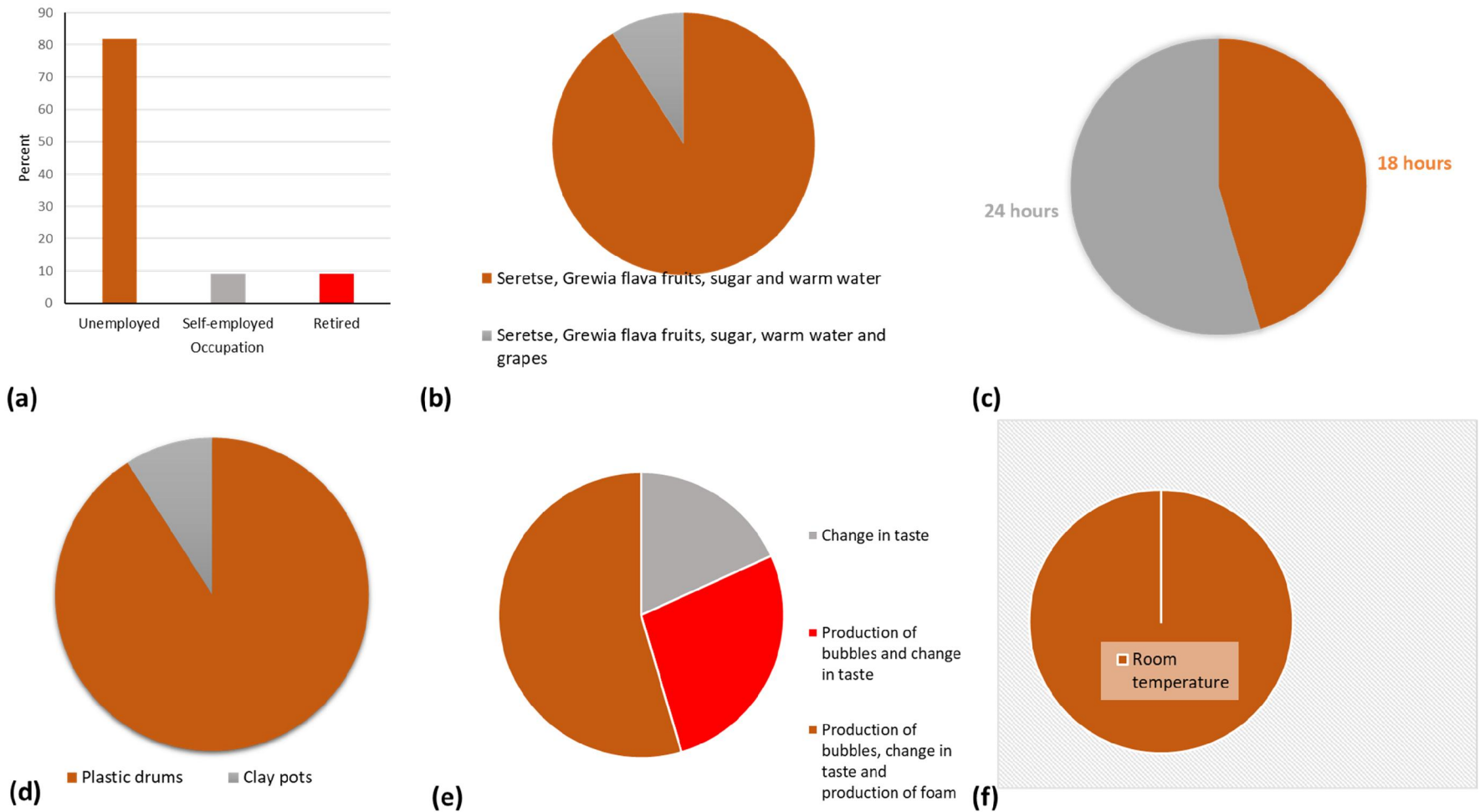


Figure 7: Results of the questionnaire on the *khadi* brewing process. (a) Livelihood of *khadi* brewers. (b) The ingredients used to produce *khadi*. (c) Fermentation duration of *khadi*. (d) Fermentation vessels used during *khadi* production. (e) Attributes used by the *khadi* brewers to indicate the end of fermentation. (f) Storage of *G. flava* fruits for *khadi* fermentation.

4.2 Physico-chemical properties of *khadi*

4.2.1 Ethanol content of *khadi*

The ethanol concentration among *khadi* ranged from 0.75 ± 0.02 to 8.73 ± 0.13 % (v/v) (Figure 8). The highest ethanol content was recorded from Maun 1 whereas Maun 3 had the lowest ethanol content. On average, ethanol content of *khadi* was 5.27 ± 2.02 % (v/v). This value was higher than the average, 3.69 % (v/v), previously reported by Mapitse et al., (2014). Commercial beers range from 3 to 6 % (v/v) (Bamforth, 2002). The diversity of fermenting yeasts among *khadi* samples could be one of the main reasons of the discrepancies of amounts of ethanol accumulated. Species from the genus *Saccharomyces* produce higher amounts of ethanol as compared to other genera and their non-*Saccharomyces* counterparts (Hagman et al., 2013). In addition, the brewing of *khadi*, which is carried out in non-standard conditions such as the use of uncontrolled and partially aerobic fermenting drums could be another factor. This is because oxygen partial pressures determine the alcoholic fermentation patterns (Merico et al., 2009). Lower oxygen partial pressures increases the glycolytic flux towards a fermentative metabolism whereas the opposite is true for environments with high oxygen concentrations (Piškur et al., 2006; Pronk et al., 1996). It is also important to note that fermentation time was not standardized in the brewing of *khadi*. Such discrepancies would lead to variations in ethanol concentration in the brew at the end of fermentation. Yeasts accumulate and consume, a characteristic of fermenting yeasts described by (Rozpedowska et al., 2011), suggest that if fermentation is not stopped upon exhaustion of fermentable carbon sources and in the presence of oxygen as the final electron acceptor, the yeasts will consume the accumulated ethanol. Since the timing of end of fermentation was not prioritized or standardized this could have revealed a batch-to- batch discrepancies in ethanol concentrations for brews. It

should also be noted that ethanol accumulates incrementally during brewing thus the amount of ethanol during the perceived end of fermentation is actually stage-specific.

4.2.2 pH of khadi, an important factor in preservation of the brew

The pH of the *khadi* samples ranged from 2.64 to 3.62 (Table C3 and Figure 8). Letlhakane 2 sample was the least acidic (pH 3.62) as compared to all other samples whereas sample Palapye 2 was the most acidic with a pH of 2.64. On average pH of *khadi* samples was 3.1 ± 0.31 . In comparison to other traditional beverages such as *tej* and palm wine, *khadi* was found to be highly acidic. To be specific, *tej* was reported to have a pH of 3.56 to 4.45 (Yohannes et al., 2013) and palm wine with pH of 4.0 to 5.5 (Karamoko et al., 2012). These results are in agreement with previous studies by Mapitse et al., (2014) who reported an average pH of 3.08 ± 0.15 . In comparison to most commercial beers, with a pH of 4 - 5, *khadi* is acidic. pH influences the overall physiological characteristics of alcoholic beverages such as taste, color and biological stability. Most importantly, pH also strongly influences sanitation of the product, an attribute important for increasing shelf life of the brew (Jukić et al., 2018). Other than the presence of ethanol, the acidic pH is likely the other reason why they were no bacteria isolated from *khadi* because bacteria are known to be sensitive to highly acidic conditions (Chelule et al., 2010; Hirshfield et al., 2003; Yu et al., 2010). Although there are other factors such as mineral content and titratable acidity among others that should be considered to make valid conclusions on the effects of acidic brew, the information on the relative acidity of the brew would be important for development of *khadi* commercialization.

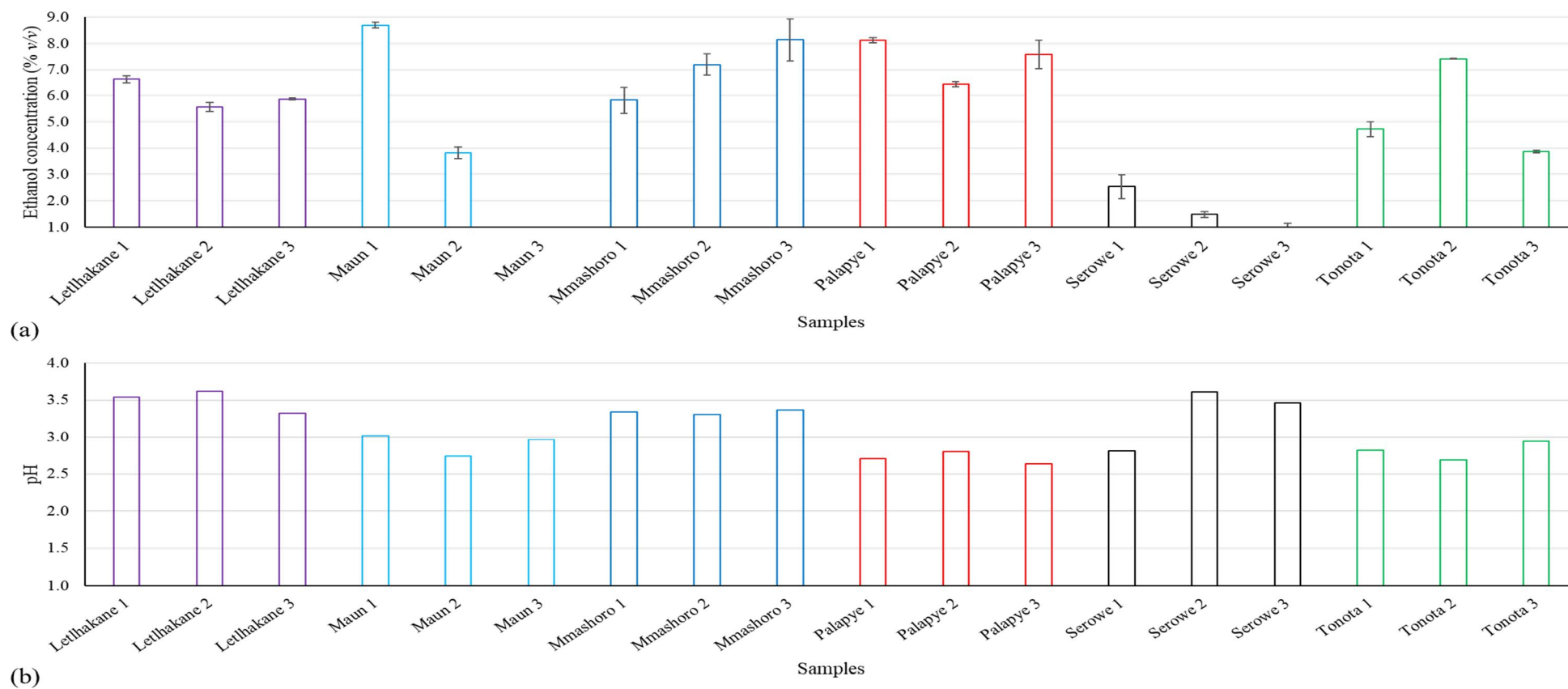


Figure 8: The physico-chemical properties of the *khadi* samples. (a) Variable average ethanol content and (b) average pH of the *khadi* samples used in the study. Color code: purple = Letlhakane, sky blue = Maun, navy blue = Mmashoro, red = Palapye, Black = Serowe and green = Tonota

4.3 Fermentative microbiota from *G. flava* fruits and *khadi*

4.3.1 Abundance of microbiota isolated from *G. flava* fruits

G. flava fruits play an important role in *khadi* fermentation as they are a possible source of fermenting microorganisms, fermentable sugars and other growth factors. For this reason, we enumerated the yeasts and bacteria found on *G. flava* fruits. It was noted that in each area there was a single supplier of the *G. flava* fruits and hence only one batch of fruits per town was used for investigation. The yeast colonies on the *G. flava* fruits ranged from 0 to 2×10^3 CFUs/mL with their average being 1×10^3 CFUs/mL while bacterial colonies ranged from 2×10^3 to 4×10^3 CFUs/mL with an average of 3.5×10^3 CFUs/mL (Table 6). The colonization of fruits by bacteria and yeasts is in agreement with studies described elsewhere (Kurtzman et al., 2011) but the low number of colonies observed could be attributed to the processing of fruits. The storage of fruits before fermentation could have created a selective environment for proliferation of a few yeasts and bacteria with specific attributes that allow them to survive in a new environment (Fleet, 2003). Drying of fruits lowers the water activity needed for microbial survival thus reducing the number of microbes (Beuchat et al., 2013). This means that grapes will have more microbes as compared to raisins.

Table 6: Abundance of yeasts and bacteria isolated from the ripened sun-dried *G. flava* fruits samples expressed in $\log_{10}$ CFU/mL (colony forming units per millimeter).

Sample	Log ₁₀ CFU/mL	
	Yeasts	Bacteria
Lethakane	3.0	3.6
Maun	3.0	3.5
Mmashoro	0	3.6
Palapye	3.3	3.3
Serowe	3.0	3.6
Tonota	3.0	3.6
Average	2.6	3.5

4.3.2 Abundance of microbiota isolated from khadi

After identification of yeasts and bacteria on fruits we sought to investigate the diversity of bacteria and yeasts after fermentation. Interestingly, there were no bacteria in all *khadi* samples investigated. On the other hand, yeasts were isolated from all the brews. This observation was not surprising, as yeasts are known to engineer the environment that favors their proliferation through fermentation. Alcoholic fermentation is a niche reconstruction lifestyle of the Crabtree positive yeasts which creates heat, CO₂ and ethanol to inhibit sensitive competitors such as bacteria (Dashko et al., 2014; Goddard, 2008; Rozpedowska et al., 2011). Crabtree positive yeasts tend to ferment even in the presence of excess oxygen and a fermentable carbon source (Dashko, Zhou, Compagno, & Pishkur, 2014). In the presence of excess sugar and oxygen, a few microorganisms, for example, some yeasts from the *Saccharomycetaceae* family, generate ATP through a respiratory or fermentative metabolism or both pathways concurrently, thus known as respiro-fermentative yeasts (De Deken, 1966).

The total number of yeasts on the *khadi* samples ranged from $5.12 \pm 0.09 \log_{10}$ CFUs/mL (Mmashoro) to $6.41 \pm 0.22 \log_{10}$ CFUs/mL (Palapye) (Figure 7). On average, $5.6 \pm 0.57 \log_{10}$ CFUs/mL of yeasts were recorded. Using one-way ANOVA at a confidence interval of 95 %, we noted that there was no significant difference in concentrations of yeasts at the end of fermentation among the samples.

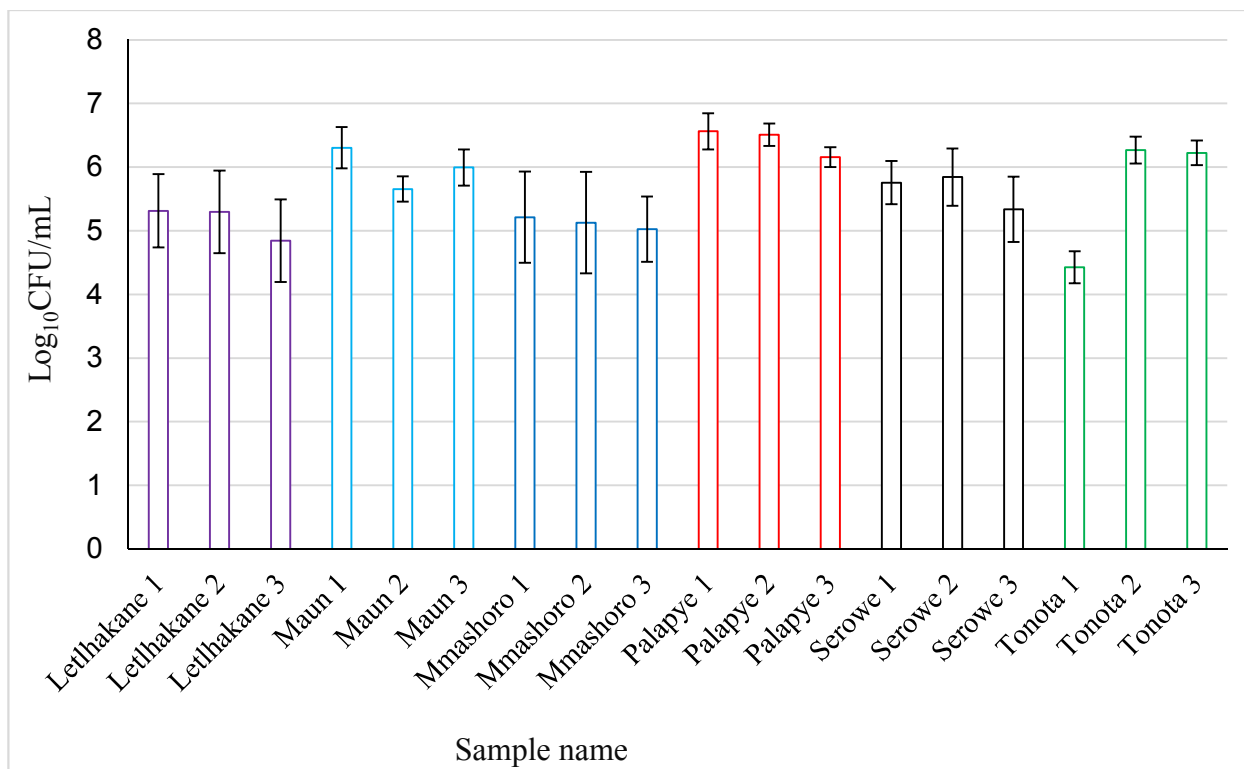


Figure 9: Variability of concentrations of yeast (log₁₀ CFU/mL) isolated from *khadi* samples from different parts of the Central and Northern Botswana. Six samples were collected in triplicate and analyzed individually. Color code: purple = Letlhakane, sky blue = Maun, navy blue = Mmashoro, red = Palapye, Black = Serowe and green = Tonota.

4.4 Investigation of bacterial isolates from *G. flava* fruits

4.4.1 Diversity of bacterial isolates

Bacteria are known to be sympatric with yeasts inhabiting sugar rich environments such as ripening fruits (Barata et al., 2012; Zhou et al., 2017a). Investigation of bacteria found on *G. flava* fruits yielded a total of 21 genetically diverse bacterial isolates (Table 7). *Bacillus* species dominated the isolates accounting for 76.2 % of the identified isolates (16/21). The dominance of *Bacillus* species was not surprising as they are ubiquitous microbes that are common in soil, air, dust, water as well as dried and processed foods of plant origin

(Stenfors Arnesen et al., 2008). Perhaps methods used to process fruits could have created an environment that selected for *Bacillus* spp. In addition, the limited diversity of bacteria skewed towards the *Bacillus* species suggests that these species could be responsible for ecosystem modification. For example, *B. velezensis* is a well-known producer of antibacterial secondary metabolites (Kim et al., 2017). The isolation of *Bacillus* spp. known for food poisoning and causing illness in human beings such as *B. cereus*, *B. subtilis*, *B. amyloliquefaciens* and *B. thuringiensis* (André et al., 2017; de Blackburn and McClure, 2009) is a cause of concern but the concern can be overlooked as none of these were isolated from the *khadi* samples. In addition, *Acinetobacter lwoffii* could also be another bacteria of concern as the genus *Acinetobacter* comprises of multiple nosocomial opportunistic pathogens that cause pneumonia (Margolin et al., 2016), but the bacterial isolates did not survive the fermentation process.

Table 7: Identity of bacteria isolated from *Grewia flava* fruits based on D1/D2 region of the 16S rDNA.

Species Name	Isolate Number	Collection Number	Accession Number	Identity (%)
<i>Acinetobacter lwoffii</i>	MMB4	Mmashoro	KF818633.1	99
<i>Acinetobacter lwoffii</i>	SB2	Serowe	KF818633.1	99
<i>Bacillus amyloliquefaciens</i>	PB1	Palapye	MG892875.1	99
<i>Bacillus cereus</i>	TB3	Tonota	AB523744.1	99
<i>Bacillus cereus</i>	LB4	Letlhakane	MG021182.1	99
<i>Bacillus methylotrophicus</i>	SB1	Serowe	KM659219.1	99
<i>Bacillus oleronius</i>	LB1	Letlhakane	KY773585.1	98
<i>Bacillus pichinotyi</i>	PB2	Palapye	MG705701.1	98
<i>Bacillus amyloliquefaciens</i> subsp. <i>plantarum</i>	MMB2	Mmashoro	JN661699.1	99
<i>Bacillus</i> spp.	MAB3	Maun	KF646681.1	99
<i>Bacillus</i> spp.	TB4	Tonota	KT443870.1	99
<i>Bacillus thuringiensis</i>	SB3	Serowe	KY910253.1	99
<i>Bacillus thuringiensis</i>	LB3	Letlhakane	KY495218.1	99
<i>Bacillus simplex</i>	LB2	Letlhakane	AB547125.1	99
<i>Bacillus subtilis</i>	MMB3	Mmashoro	MH261154.1	99
<i>Bacillus subtilis</i>	SB4	Serowe	MH261154.1	98
<i>Bacillus subtilis</i>	TB1	Tonota	MH261154.1	99
<i>Bacillus velezensis</i>	MMB1	Mmashoro	MH000677.1	99
<i>Desemzia incerta</i>	TB2	Tonota	KF712891.1	99
<i>Exiguobacterium indicum</i>	MAB1	Maun	MH819520.1	93
<i>Staphylococcus saprophyticus</i>	MAB2	Maun	CP022093.2	99

4.4.2 Biochemical characterization of the bacteria isolates

The biochemical test results showed that 18 isolates were catalase positive, 17 isolates were Gram positive, 11 isolates were oxidase positive, 9 isolates were methyl red positive, 9 isolates were lactose fermentation positive, 7 isolates were Voges-Proskauer positive, 6 isolates were urease positive and all the isolates were indole negative (Table 8). The Gram staining results showed that the majority of the isolates were Gram positive in agreement to the molecular identification results while TB4 and LB1 were Gram negative and were identified as *B. oleronius* (Szkaradkiewicz et al., 2012). Even though staining Gram-negative, *B. oleronius* has Gram-positive cell wall components shared amongst all *Bacillus* species (Owusu-Darko et al., 2017). To identify those organisms that hydrolyze urea, the urease test was performed and the results show that only 5 isolates can hydrolyze urea as seen in Table 8. This means only these isolates have the urease enzyme thus allowing them to hydrolyze urea producing carbon dioxide and ammonia (Yarrow, 1998). If these organisms are allowed or capable of growing in an alcoholic beverage, they would spoil the quality of the beverage due to the metabolites they produce such as ammonia and indole (Comi, 2016). Table 8 summarizes the rest of the characterization results of the bacterial isolates. Some bacteria have the ability to perform mixed acid fermentation of glucose producing a complex mixture of acids, particularly lactate, acetate, succinate and formate as well as ethanol and equal amounts of H₂ and CO₂ causing the medium to acquire an acidic pH (Tassadaq et al., 2013). The results showed that 9 isolates were methyl red positive meaning their presence in *khadi* could have possibly make the beverage acidic.

Table 8: Biochemical characterization and colony pigmentation of bacterial isolates from *G. flava* fruits.

Isolate name	Isolate code	Gram staining	URE	IND	LAC	VP	CAT	MR	OXI	Colony colour
<i>Bacillus subtilis</i>	TB1	+	-	-	+	+	+	+	-	Opaque white
<i>Desemzia incerta</i>	TB2	+	+	-	+	-	+	+	+	Opaque yellow
<i>Bacillus cereus</i>	TB3	+	-	-	-	-	-	-	-	Pale yellow
<i>Bacillus</i> spp.	TB4	-	-	-	-	-	-	-	-	Pale yellow
<i>Bacillus methylophilus</i>	SB1	+	-	-	+	-	+	-	+	Opaque white
<i>Acinetobacter lwoffii</i>	SB2	-	-	-	-	-	+	-	+	Opaque white
<i>Bacillus thuringiensis</i>	SB3	+	-	-	+	+	+	+	+	Opaque white
<i>Bacillus subtilis</i>	SB4	+	+	-	+	+	+	+	+	Opaque white
<i>Bacillus amyloliquefaciens</i>	PB1	+	-	-	+	+	+	-	+	Cream white
<i>Bacillus pichinotyi</i>	PB2	+	-	-	-	-	+	+	+	Cream white
<i>Exiguobacterium indicum</i>	MAB1	+	-	-	-	+	+	+	-	Pale yellow
<i>Staphylococcus saprophyticus</i>	MAB2	+	+	-	+	-	+	+	-	Opaque white
<i>Bacillus</i> spp.	MAB3	+	+	-	+	+	+	+	+	Opaque white
<i>Bacillus amyloliquefaciens</i> subsp. <i>plantarum</i>	MMB2	+	-	-	+	-	+	-	-	Dark yellow
<i>Bacillus velezensis</i>	MMB1	+	-	-	-	-	+	-	-	White
<i>Bacillus oleronius</i>	LB1	-	-	-	-	-	+	-	-	Yellow
<i>Bacillus simplex</i>	LB2	+	-	-	-	+	+	+	+	Opaque white
<i>Bacillus thuringiensis</i>	LB3	+	-	-	+	+	+	+	+	Opaque white
<i>Bacillus cereus</i>	LB4	+	-	-	-	-	-	-	-	Pale yellow
<i>Bacillus subtilis</i>	MMB3	+	-	-	+	+	+	+	+	Opaque white
<i>Acinetobacter lwoffii</i>	MMB4	-	-	-	-	-	+	-	+	Opaque white

*Note: URE – urease test, IND – indole test, LAC – lactose test, VP - Voges-Proskauer test, CAT – catalase test, MR – methyl red test and OXI – oxidase test

4.5 Investigation of yeast isolates from *G. flava* fruits

4.5.1 Diversity of yeast isolates

After the observation that there were a number of yeasts with different morphologies, we sought to identify yeasts by sequencing of the 26S rDNA. A total of 6 species dominated the fruit samples namely; *Aureobasidium leucospermi*, *A. melanogenum*, *Naganishia diffluens*, *Aureobasidium* spp., *A. pullulans* and *S. cerevisiae* (Table 9). *Aureobasidium* spp. was the most predominant species in all samples. Different *Aureobasidium* spp. have been reported to be among the non-*Saccharomyces* yeasts known to be involved in spontaneous fermentations of wine, beer, tequila, mezcal and cachaça (Varela, 2016a) and hence they could be contributors of alcoholic fermentation of *khadi*. In addition, the isolation of *Aureobasidium pullulans* from other wild fruits and their fermented products reported elsewhere (Nyanga et al., 2007), further suggest that this dominant genus could be involved in the fermentation of *khadi*. Some of the isolates from the fruits have an unknown contribution to the fermentation of *khadi* such as *Naganishia diffluens* which has been reportedly found to colonize flowers of edible fruit plants (such as pomegranate, mango, guava and lemon) (Moubasher et al., 2018). It is not surprising that a well-known fermenting species of yeast inhabiting sugar rich environments such as wild fruits, *S. cerevisiae*, was only isolated from Letlhakane samples because *S. cerevisiae* is naturally initially rare on fruit ecosystems, but it eventually dominates the niche after engineering the ecosystem through fermentation (Goddard, 2008; Ponzzes-Gomes et al., 2014). Therefore,

it is likely that *S. cerevisiae* could have been present in other fruit samples but was not isolated.

It should be noted that more microbial diversity could have been identified if more of the *G. flava* fruits were used instead of only 2g. This could have limited the number and types of microbes isolated as compared to if more fruits were used for the isolation of the microbes thus the limitation of the selected method.

Table 9: Identity of yeast isolated from *G. flava* fruits

Species name	Isolate number	Collection number	Accession number	Identity (%)
<i>A. leucospermi</i>	D2	Palapye	KX893326.1	99
<i>A. melanogenum</i>	D3	Serowe	MF370933.1	99
<i>A. pullulans</i>	D6	Tonota	MF979210.1	99
<i>Aureobasidium spp.</i>	D5	Maun	KT361586.1	99
<i>Naganishia diffluens</i>	D4	Palapye	KU316762.1	99
<i>S. cerevisiae</i>	D7	Letlhakane	HQ108377.1	99

4.6 Yeasts as major contributors in fermentation of *khadi*

4.6.1 Diversity of yeasts

Mainly Saccharomycotina and a few Basidiomycota yeasts were isolated from all the brews. This observation was not surprising, as Saccharomycotina yeasts are known to engineer the environment that favors their proliferation through fermentation (Goddard, 2008). Alcoholic fermentation is a niche reconstruction lifestyle of Crabtree positive yeasts which creates heat, CO₂ and ethanol to inhibit the growth of competitors (Dashko et al., 2014; Goddard, 2008; Rozpedowska et al., 2011). The limited diversity of yeasts on the dried fruits in comparison to the wide diversity of bacteria prompted further investigation to

establish which microorganisms could be responsible for the fermentation of *khadi*. A total of 74 yeast isolates with a wide phylogenetic diversity were recorded from different *khadi* samples (Table 10, Figure 10). The isolates belonged to 13 yeast species (Table 11). Interestingly, most of those yeasts were not isolated from the ripened sun-dried fruits. There are two possibilities to explain this; (i) there was a source of yeasts other than those from the wild fruits such as fermentation vessels (Aouizerat et al., 2019) , (ii) there were yeasts that were rare on the fruit surfaces and were not identified due to the limitation of culture-based techniques (Baselga et al., 2017; Wade, 2002). Interestingly, no bacteria were isolated from *khadi* and this might have been due to the possible temperature bias used for the incubation of LAB.

S. cerevisiae dominated the isolates (51.4%) followed by *Candida ethanolica* (12.2%) and *Saccharomyces ludwigii* (10.8%) (Tables 10 and 11). The dominance of *S. cerevisiae* at the end of the alcoholic fermentation, despite the dominance of non-conventional yeasts and bacteria, during the early stages of fermentation is well documented (Goddard, 2008; Ponzzes-Gomes et al., 2014; Varela, 2016b). These results suggest that *khadi* is a product of mixed-culture fermentation with phylogenetically diverse yeasts dominated by *S. cerevisiae* (Table 10).

Mixed-culture fermentations made up of conventional and non-conventional yeasts leads to distinctive aromatic profiles of alcoholic beverages (Andorrà et al., 2012; Ciani et al., 2009; Dashko et al., 2015a; Gamero et al., 2016; Lencioni et al., 2016). The production of aroma compounds in alcoholic beverages also requires non-conventional yeasts. For example, *Lachancea* spp. are well-known producers of fruity esters, which impart a characteristic fruity flavor to alcoholic brews (Cordero-Bueso et al., 2012; Porter, 2017; Romano et al., 1997). Fermentative species of the genus *Candida*, such as *C. ethanolica* and *C. sake*, also known to generate unique flavors of a brew, were isolated (Maturano et al., 2015) from

brews from Letlhakane, Tonota and Maun. However, the isolation of spoilage yeasts, such as *Brettanomyces* spp. and *Zygosaccharomyces* spp. (Alonso et al., 2015; James and Stratford, 2003; Schifferdecker et al., 2016), in samples from Serowe and Tonota, respectively, could negatively impact on the quality of a spontaneously fermented brew. *Brettanomyces bruxellensis* is known to be both a spoilage yeast and good fermenting yeast. This yeast has been isolated from wines that have characteristically developed unpleasant and distinctive aromas due to the production of volatile phenolic compounds and diacetyl (Chatonnet et al., 1995; Hittinger et al., 2015; Licker et al., 1998; Schifferdecker et al., 2016). These unpleasant and distinctive aromas production is due to the production phenols exceeding the sensory threshold 16-fold. In contrast, *Brettanomyces* spp. have been used for production of Lambic beers where they can produce ethyl esters which contribute to the overall fruity or floral character of the beverage at a lower sensory threshold (Capece et al., 2018; Schifferdecker et al., 2014).

Zygosaccharomyces bailii is a wine spoilage yeast associated with grapes whose spoilage is characterized by generation of taints, odors, off-flavors, development of hazes, and excessive gas production (Barata et al., 2012; James and Stratford, 2003). Literature suggests that *Z. bailii* can improve the flavor complexity of alcoholic beverages either used individually or as a mixed culture with *S. cerevisiae* producing flavor compounds such as alcohols, acids, esters, ketones and aldehydes (Xu et al., 2017). A concern for spontaneously fermented brews is the presence of clinically relevant species. *Pichia kudriavzevii* (also known as *Candida krusei* (Douglass et al., 2018)) was the only potentially opportunistic pathogen (Douglass et al., 2018) that was isolated and it was found in a Letlhakane sample. However, *P. kudriavzevii* also has good fermentation characteristics and it has been previously isolated from *taruba*, grapes and *masau* fruits (Nyanga et al., 2013b; Ponzzes-Gomes et al., 2014; Ramos et al., 2015).

One of the expected features of a fermented brew is the presence of *Saccharomyces* yeasts. The phylogenetic tree based on the Hasegawa–Kishino–Yano model in Figure 10 shows the relationship among the *S. cerevisiae* isolates isolated from the *khadi* samples. The tree (Figure 10) suggests that *S. cerevisiae* isolates isolated from different locations are diverse as they cluster into 7 different groups. The results suggest that some brewers could be using commercial ale, baker's, lager and/or wine strains instead of spontaneous fermentation. For example, Group 7 (Figure 10) isolates clustered with a control commercial baker's yeast, which is readily available in local retail shops. In addition, it was noted that some isolates clustered with commercial ale and wine yeasts as shown in Group 4, which are not available in the local supermarkets of Botswana, while two isolates from Maun (MA6) and Letlhakane (L3) clustered in a separate group with the commercial lager yeast. These clusters suggest that commercial yeasts strains have been used as a starter culture. Brewers prefer the addition of starter cultures to circumvent the negative outcomes of spontaneous fermentation, which produces an inconsistent quality of fermented products. Contrastingly, we noted that *S. cerevisiae* strain isolated from *G. flava* fruits (from Letlhakane) clustered with *S. cerevisiae* isolates from *khadi* from Serowe and Mmashoro in Group 5, suggesting that use of spontaneous fermentation could be popular among these two locations. Use of dried fruits from the same supplier in these three locations could also be a possible explanation for the isolation of yeasts that were closely related to isolates from the fruits.

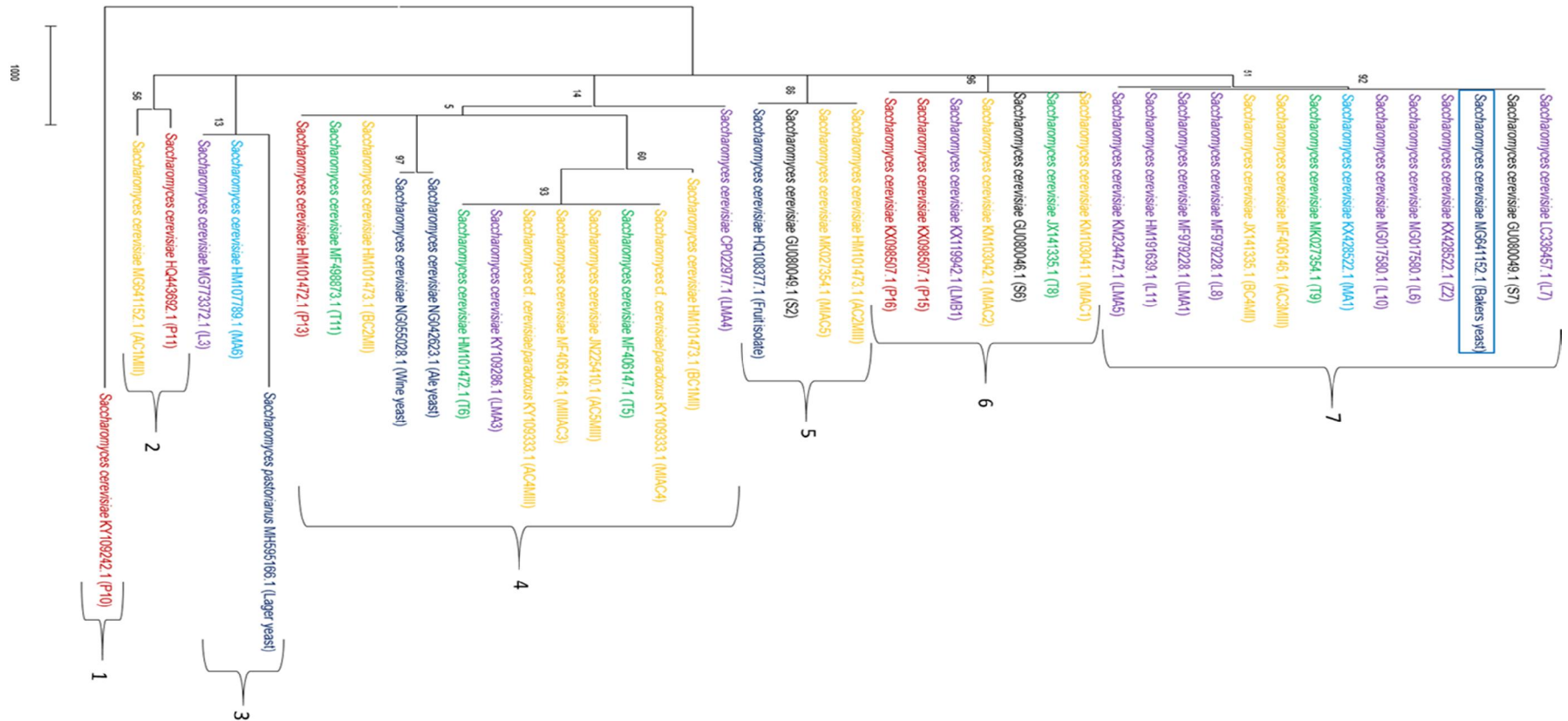


Figure 10: Maximum likelihood analysis of the D1/D2 domains of 26S rDNA of *Saccharomyces* yeasts isolated from *khadi* samples. The evolutionary relationship between isolates was inferred using the maximum likelihood method based on the Hasegawa–Kishino–Yano model (Tamura and Nei, 1993). Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2018). Color code: purple = Letlhakane, sky blue = Maun, yellow = Mmashoro, red = Palapye, Black = Serowe, green = Tonota and navy blue = Controls.

Table 10: Identity of yeast isolates from the *khadi* samples based on D1/D2 domains of 26S rDNA.

Species Name	Isolate Number	Collection Number	Accession Number	Identity (%)
<i>Saccharomyces</i> yeasts				
<i>Saccharomyces cerevisiae</i>	Z2	Letlhakane 1	KX428522.1	99
<i>Saccharomyces cerevisiae</i>	L3	Letlhakane 1	MG773372.1	99
<i>Saccharomyces cerevisiae</i> (2)	L6 and L10	Letlhakane 2	MG017580.1	99
<i>Saccharomyces cerevisiae</i>	L7	Letlhakane 2	LC336457.1	99
<i>Saccharomyces cerevisiae</i>	L8	Letlhakane 3	MF979228.1	99
<i>Saccharomyces cerevisiae</i>	L11	Letlhakane 3	HM191639.1	99
<i>Saccharomyces cerevisiae</i>	LMA1	Letlhakane 2	MF979228.1	99
<i>Saccharomyces cerevisiae</i>	LMA3	Letlhakane 2	KY109286.1	99
<i>Saccharomyces cerevisiae</i>	LMA4	Letlhakane 1	CP022977.1	99
<i>Saccharomyces cerevisiae</i>	LMA5	Letlhakane 2	KM234472.1	99
<i>Saccharomyces cerevisiae</i>	LMB1	Letlhakane 2	KX119942.1	99
<i>Saccharomyces cerevisiae</i>	MA1	Maun 1	KX428522.1	99
<i>Saccharomyces cerevisiae</i>	MA6	Maun 2	HM107789.1	99
<i>Saccharomyces cerevisiae</i>	AC1MIII	Mmashoro 3	MG641152.1	99
<i>Saccharomyces cerevisiae</i> (2)	AC2MIII and BC1MII	Mmashoro 3 and 2	HM101473.1	99
<i>Saccharomyces cerevisiae</i>	AC3MIII	Mmashoro 3	MF406146.1	99
<i>Saccharomyces cerevisiae</i>	AC5MIII	Mmashoro 3	JN225410.1	99
<i>Saccharomyces cerevisiae</i>	BC4MII	Mmashoro 2	JX141335.1	99
<i>Saccharomyces cerevisiae</i>	MIAC1	Mmashoro 1	KM103041.1	99
<i>Saccharomyces cerevisiae</i>	MIAC3	Mmashoro 1	MF406146.1	99
<i>Saccharomyces cerevisiae</i>	MIAC2	Mmashoro 1	KM103042.1	99
<i>Saccharomyces cerevisiae</i>	MIAC5	Mmashoro 1	MK027354.1	99
<i>Saccharomyces cerevisiae</i>	P10	Palapye 1	KY109242.1	99
<i>Saccharomyces cerevisiae</i>	P11	Palapye 1	HQ443692.1	99
<i>Saccharomyces cerevisiae</i>	P13	Palapye 3	HM101472.1	99
<i>Saccharomyces cerevisiae</i> (2)	P14 and P16	Palapye 2	KX098507.1	99
<i>Saccharomyces cerevisiae</i>	P17	Palapye 3	GU080046.1	99
<i>Saccharomyces cerevisiae</i> (2)	S2 and S7	Serowe 1	GU080049.1	99
<i>Saccharomyces cerevisiae</i>	S6	Serowe 2	GU080046.1	99
<i>Saccharomyces cerevisiae</i>	T5	Tonota 1	MF406147.1	99
<i>Saccharomyces cerevisiae</i>	T6	Tonota 1	HM101472.1	99
<i>Saccharomyces cerevisiae</i>	T8	Tonota 1	JX141335.1	99
<i>Saccharomyces cerevisiae</i>	T9	Tonota 2	MK027354.1	99
<i>Saccharomyces cerevisiae</i>	T11	Tonota 2	MF498873.1	99
<i>Saccharomyces</i> cf. <i>cerevisiae</i> /paradoxus (2)	AC4MIII and MIAC4	Mmashoro 1 and 3	KY109333.1	99
Non-<i>Saccharomyces</i> yeasts				
<i>Brettanomyces bruxellensis</i>	S5	Serowe 2	KY107600.1	99
<i>Candida ethanolica</i>	LMB2	Letlhakane 3	KY283163.1	99
<i>Candida ethanolica</i> (2)	LMB4 and LMC1	Letlhakane 1	FM180545.1	99
<i>Candida ethanolica</i>	LMC4	Letlhakane 1	JX880409.1	99
<i>Candida ethanolica</i> (5)	T10, T14 T15, T17, T18	Tonota 2 and 3	JX880409.1	99
<i>Candida sake</i> (2)	MA2 and MA3	Maun 1	JX880410.1	99
<i>Curvibasidium pallidicorallinum</i>	MA7	Maun 3	JX188149.1	99
<i>Pichia kudriavzevii</i>	L13	Letlhakane 3	AY529504.1	99
<i>Pichia kudriavzevii</i>	S8	Serowe 3	AY529504.1	91
<i>Lachancea fermentati</i>	MA4	Maun 2	KY108224.1	99
<i>Lachancea fermentati</i>	MA8	Maun 3	KM234440.1	99
<i>Lachancea fermentati</i>	P18	Palapye 3	KM234440.1	99
<i>Pichia kudriavzevii</i>	L1	Letlhakane 1	KM234442.1	99
<i>Pichia kudriavzevii</i> (2)	L2 and L4	Letlhakane 2	KF214396.1	99

<i>Pichia manshurica</i>	P15	Palapye 2	MK034750.1	99
<i>Rhodotorula nothofagi</i>	Z1	Letlhakane 1	KJ794722.1	99
<i>Saccharomycodes ludwigii</i> (2)	L9 and L12	Letlhakane 3	FM180540.1	99
<i>Saccharomycodes ludwigii</i> (2)	S1 and S4	Serowe 1	FM180540.1	99
<i>Saccharomycodes ludwigii</i> (4)	T7, T13, T12, T16	Tonota 1 and 2	FM180540.1	99
<i>Schizosaccharomyces pombe</i>	P8	Palapye 2	KY296084.1	99
<i>Zygosaccharomyces bailii</i>	T1	Tonota 1	GU080052.1	99
<i>Zygosaccharomyces bailii</i>	T4	Tonota 1	KY296086.1	99

Note: The number in the bracket shows strains with the same accession number from the same location.

Table 11: The number and percentage of yeast isolates from *khadi* samples.

Species	Number of isolates sequenced	%	Letlhakane	Maun	Mmashoro	Palapye	Serowe	Tonota
<i>Saccharomyces cerevisiae</i>	38	51.4	12	2	10	6	3	5
<i>Candida ethanolica</i>	9	12.2	4					5
<i>Saccharomycodes ludwigii</i>	8	10.8	2				2	4
<i>Pichia kudriavzevii</i>	5	6.75	4				1	
<i>Zygosaccharomyces bailii</i>	2	2.7						2
<i>Candida sake</i>	2	2.7		2				
<i>Saccharomyces cf. cerevisiae/paradoxus</i>	2	2.7			2			
<i>Lachancea fermentati</i>	3	5.4		2		1		
<i>Curvibasidium pallidicorallinum</i>	1	1.4		1				
<i>Schizosaccharomyces pombe</i>	1	1.4				1		
<i>Brettanomyces bruxellensis</i>	1	1.4					1	
<i>Rhodotorula nothofagi</i>	1	1.4	1					
<i>Pichia manshurica</i>	1	1.4				1		
Total	74	100	23	7	12	9	7	16

4.6.2 The fermenting microorganism behind *khadi*

Yeasts play a pivotal role (ethanol production) in fermentation of all alcoholic beverages (Piškur et al., 2006). Although *S. cerevisiae* species were found in low frequencies on *G. flava* fruits

before fermentation, the species dominated the isolates from the fermented product, *khadi*. All samples were collected from spontaneously fermented brews or from back-slopped brews, therefore, the fermenting *Saccharomyces* spp. could be those that were introduced from the ripened sun-dried fruits or previously fermented fruits (inoculum) as well as those resident on the brewing equipment and the brown table sugar as in agreement with the following study (Boekhout and Phaff, 2003).

The diversity of *S. cerevisiae* strains isolated from *khadi* in comparison to the fruits prompted us to further investigate adulteration of the brewing process using readily available commercial *S. cerevisiae* strains sold by retail stores in Botswana. The brewers may add commercial baker's yeasts during *khadi* production to give it 'strength' and 'enhance its capacity' to intoxicate, instead of relying on spontaneous fermentations (Rudloff et al., 2012). An investigation into the genetic diversity of the *Saccharomyces* spp. using *in-silico* PCR-RFLP suggests that most of the isolates were not genetically distinct from the commercial baker's yeast (Fig 11a). To be specific, restriction fragment patterns of 67.5% of the strains of this species (27 out of 40 isolates) digested with *Hae*III matched a characteristic restriction fragment pattern of the commercial baker's yeast strain (Fig 11a and see also Appendix C). On the other hand, 13 unique restriction fragment patterns from Mmashoro (AC1MIII, AC2MIII, AC3MIII, AC5MIII, MIAC2 and MIAC3), from Palapye (P10, P14, P16 and P17) and from Letlhakane (LMA4, LMA5 and Z2) which neither matched the *S. cerevisiae* isolate from fruits nor the commercial baker's, ale, lager and wine strains were observed (Fig 11a). This suggests that there are other sources of fermenting yeasts unaccounted for in this study. One source of yeasts could be equipment used for brewing and pre-processing. Another possibility is that the yeasts were present on the fruit surfaces at lower frequencies to be isolated using the plate count method. The latter is not

surprising because different studies have shown that *S. cerevisiae* is usually found in low frequencies during the beginning of spontaneous fermentations as compared to other yeasts, but accounts for the highest densities at the end of fermentation (Dashko et al., 2014; Goddard, 2008). On the other hand, there is a possibility that some colonies that had similar macro and microscopic morphology to the *S. cerevisiae* isolate from fruits were overlooked since in an effort to minimize potentially repetitive sequencing.

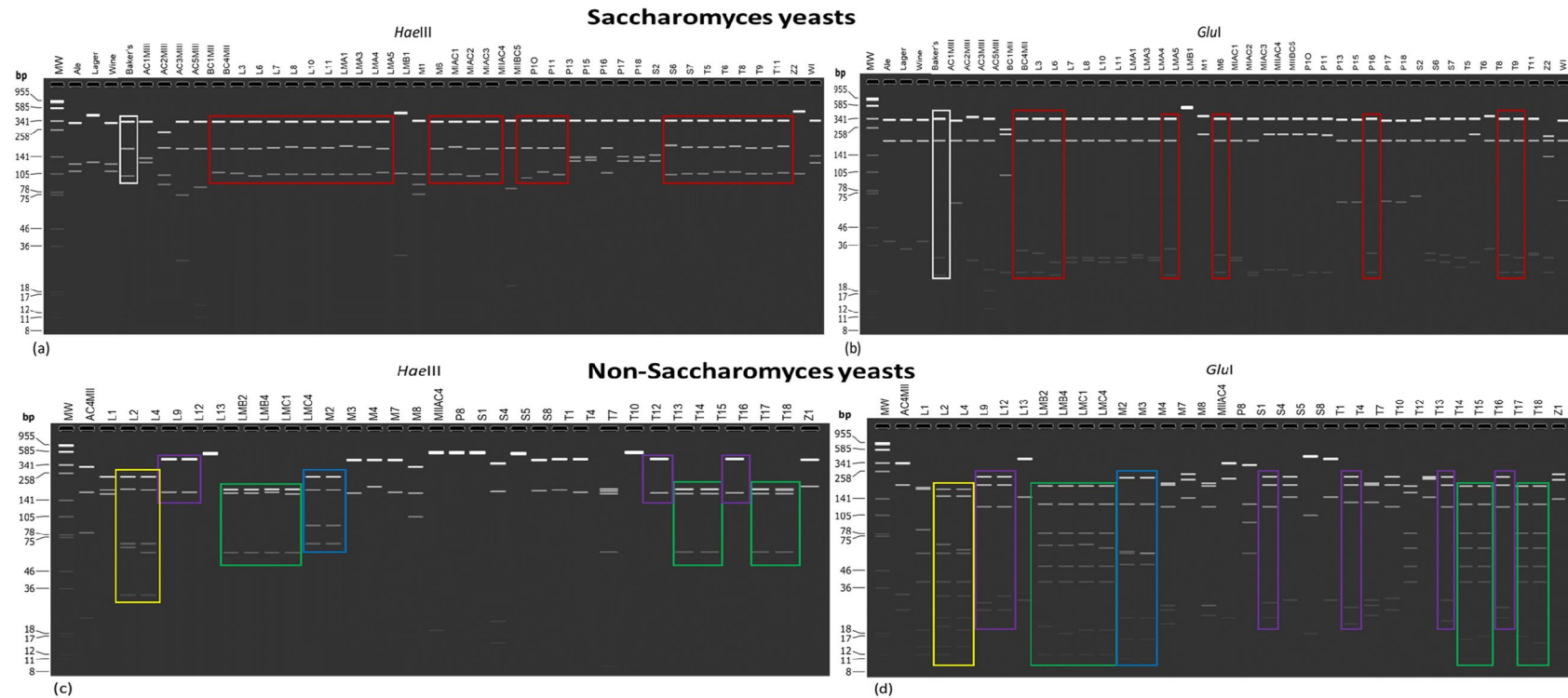


Figure 11: PCR-RFLP for yeast isolates from *khadi*. (a) and (b) show restriction patterns of *Saccharomyces* yeasts obtained using restriction enzymes *HaeIII* and *HinfI*. (c) and (d) show restriction patterns of non-*Saccharomyces* yeasts obtained using restriction enzymes *HaeIII* and *GluI*. The red shows isolate's similar to the control commercial baker's yeast (white boundary) while the WT is the wild type *S. cerevisiae* isolated from the *G. flava* fruits (Fig 11a and 11b). The red boundary shows *C. ethanolica* that have the same restriction patterns while purple shows the *Saccharomycodes ludwigii* with the same restriction patterns (Fig 11c and 11d).

Digestion with another enzyme, *GluI* suggests that there are subgroups within the strains (such as *C. ethanolica*, *C. sake*, *P. kudriavzevii* and *Saccharomycodes ludwigii*) (Figure 11b). There was no restriction fragment pattern of isolates from *khadi* that matched the pattern of the *S. cerevisiae* isolate from *G. flava* fruits. There are several possible scenarios, which can explain the absence of this strain's restriction fragment patterns after fermentation. That is, the strain could have existed in low frequencies to be picked amongst other isolates especially if back slopping was practiced because the back-slopped inocula (including the wild isolate *S. cerevisiae* isolate from the fruits) could have outcompeted it during the fermentation. In spite the clustering of some isolates with the commercial baker's yeast (Figure 10), the prevalence of strains with a distinct restriction fragment pattern from that of the commercial baker's yeast suggests that Mmashoro and Letlhakane are the only areas that rely on spontaneous fermentation for brewing of *khadi* (Appendix C). The *in-silico* PCR-RFLP results further revealed that none of the *khadi* isolates had a similar restriction fragment pattern to the selected ale and lager brewing yeasts and wine yeasts used in the industry even though there were similarities in the sequencing data (Figure 10). These yeasts are not available in the local supermarkets of Botswana as compared to the readily available baker's yeast explaining why they are not prevalent.

Non-conventional yeasts could be responsible for fermentation of *khadi* rather than conventional *Saccharomyces* yeasts alone. The fermentative lifestyle of species of the genus *Saccharomyces* is not unique among yeasts of the *Saccharomycetaceae* family (Hagman et al., 2013; Piškur et al., 2006; Rozpedowska et al., 2011) but may have coincided with the origin of angiosperms, about 125 million years ago (Hagman et al., 2013; Sun et al., 2011). Therefore, other non-conventional yeasts in the *Saccharomycetaceae* family, which existed in the same time period, are also capable of making and accumulating ethanol. In this study we identified a number of non-

conventional yeasts (Table 10) which could have also been responsible for fermentation of *khadi*. We therefore further investigated the genetic diversity using *in-silico* PCR-RFLP of non-*Saccharomyces* yeasts to reveal unique strains responsible for fermentation of different brews. We observed that among the genetically diverse yeasts, there were several unique yeasts that could be responsible for *khadi* fermentation (Figure 11c and 11d). We noted that *C. ethanolica* strains isolated from Tonota (T14, T15, T17 and T18) had matching restriction fragment pattern with *C. ethanolica* strains from Letlhakane (LMB2, LMB4, LMC1 and LMC4) from digestion with *HaeIII*. These two locations are 200 km apart and therefore sharing of the back slopped broth or dried fruits is highly unlikely although the ability of yeasts to be dispersed by insects is well documented. Therefore, this suggests that these strains could be naturally occurring on *G. flava* fruits. In agreement to our assertions, strains from a different genus, *Saccharomycodes ludwigii* from *khadi* from the same locations Tonota (T13, and T16) and Letlhakane (L9 and L12) also had a similar restriction fragment pattern when digested using *HaeIII* (Figure 11c). Similar results can be observed when running the same experiment using *GluI* (Figures 11c and 11d) as seen in Appendix C. This suggests the primary source of the fermenting yeasts could have been the fruits and not the fermentation vessels or the equipment used. It is noteworthy that non-*Saccharomyces* yeasts are not commercially available in Botswana to further suggest their role in spontaneous fermentation of *khadi*. There has been a rapid expansion of craft breweries that utilise non-*Saccharomyces* yeasts in the modern brewing industry (Alfeo et al., 2019; Callejo et al., 2017; Iattici et al., 2020). This strategy has gained special attention due to the demand for innovative and aromatically diverse beers associated with the changing lifestyle in humankind (Michel et al., 2016; Ravasio et al., 2018). Non-*Saccharomyces* yeasts have been demonstrated to produce a wide range of aromatic compounds and other metabolites that known

to improve the sensorial profiles of alcoholic beverages (Andorrà et al., 2012; Callejo et al., 2017; Dashko et al., 2015a). This work further increases the attention to exploit these yeasts to define *khadi* traits towards the development of this traditional beverage. The characterisation of these non-*Saccharomyces* yeasts (not presented in this paper) should be explored to understand how these diverse yeasts reported here could enrich and diversify aromas and flavours. Such studies would pave way into improvement of the sensory complexity of *khadi* towards its entry into regional and international markets.

4.6.3 Bioprocess development initiative

The isolation of different microorganisms from *khadi* belonging to the *Saccharomycetaceae* complex, which are possibly responsible for fermentation, is one of the crucial steps towards the development of starter cultures for the safe and hygienic bioprocessing of *khadi*. Stress tolerance tests were done to investigate the ability of the strains to be used as starter cultures. These stress tolerance tests were meant to pave the way for the commercialization of *khadi*. The tests included carbon assimilation, fermentation profiling, ethanol inhibition, osmo-tolerance, thermo-tolerance, oxidative stress and inhibition due to pH. These tests were done because there are several stresses that microbes encounter during fermentation thus, it is pertinent to select the best performing strain to be used for the bioprocess development of *khadi*.

4.6.3.1 Carbon compound assimilation

Strain selection is pivoted on the ability of yeasts to utilize different carbon sources in many industries, such as food, bioethanol and pharmaceutical (Batistote et al., 2012). In this work, we sought to ensure the ability of the isolated yeast strains to assimilate other carbon sources for use

of inexpensive and alternative carbon sources during the production. The results in Table 12 below show all the yeasts could assimilate glucose and maltose as carbon sources. All the strains could not use N-acetyl-glucosamine as carbon source, which is a monosaccharide and a derivative of glucose. This is not surprising because N-acetyl-glucosamine is structurally very similar to glucose, but it cannot be metabolized by yeast thus it provides a stable signal that glucose is present in the culture, and triggers glucose repression (Jarosz et al., 2015). A typical industrial fermentation media contains starch as well as sucrose, maltose, fructose, glucose, and other oligosaccharides (Donalies et al., 2008; Randez-Gil et al., 2003) hence the most appropriate production strain should be able to efficiently utilize a wide range of carbon sources (Zhou et al., 2017b). The utilization of glucose is to be expected as the glucose was used in the selection media.

Table 12: Carbon assimilation of the yeast isolates from *G. flava* fruits and *khadi* samples.

Substrate	D2	D3	D4	MA1	MA2	MA4	MA7	P8	S1	S5	T1	T17	Z1
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	-	+	+	+	+	+	+	+	+	+	-
2KG	-	+	-	+	-	+	+	-	-	+	-	+	-
L-Arabinose	-	+	-	+	-	+	-	-	-	-	+	+	-
D-Xylose	-	+	-	+	-	+	-	+	-	+	-	-	-
Adonitol	+	+	-	+	-	+	-	-	-	-	-	-	-
Xylitol	-	+	-	+	-	+	-	-	-	-	+	-	-
D-Galactose	+	+	+	+	+	+	-	+	+	-	+	+	+
Inositol	-	+	-	+	+	+	+	-	-	-	-	-	-
D-Sorbitol	-	+	-	+	-	+	+	-	-	+	-	-	-
MDG	-	+	-	+	-	+	+	-	-	+	-	-	-
NAG	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Cellobiose	+	+	-	+	-	+	+	+	-	-	+	+	-
D-Lactose	-	+	+	+	-	+	-	-	-	-	-	-	-
D-Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Saccharose	+	+	+	+	+	+	+	+	+	+	+	+	-
D-Trehalose	-	+	+	+	-	+	-	-	-	+	+	-	-
D-Melezitose	+	+	+	+	-	+	-	-	-	+	+	-	-
D-Raffinose	+	+	+	+	-	+	+	+	+	+	+	+	-

Notes: - = no growth, + = growth, 2KG = Calcium 2ketotetraGluconate, MDG = Methyl- α -Glucopyranoside and NAG = N-Acetyl-Glucosamine.

Khadi brewing process involves the use of local commercial brown sugar from local supermarkets. To investigate the possibility of using an inexpensive carbon source as an alternative for sucrose, the isolates were grown on different carbon sources namely maltose, fructose, lactose and sucrose at different concentrations as a control. Thus, the aim of this

experiment was to check if the isolated strains can utilize other carbon sources other than sucrose. Most of the isolates grew well at 10 % sucrose except D4 which is an isolate from *G. flava* fruits thus the result is not that much surprising as the fruit is not expected to have such high level of sucrose. The isolates D2, D4 and MA2 did not grow on 20 % sucrose while MA1, P8 and T1 showed poor growth (Figure 12). The control baker's yeast strain had an average growth at 20 % sucrose which is to be expected as the strain is used in high sugar concentration doughs with an average sucrose level of approximately 30 % (Verstrepen et al., 2006).

The yeast isolates were also tested for their utilization of different sugars namely maltose, fructose and lactose at a 2 % level. The isolates D3 and control baker's yeast strain grew very well on the lactose media while D2, D4, MA4 and Z1 showed a poor growth. Lactose is a disaccharide, made up of galactose and glucose, whose utilization depends on the presence of the *LAC4* genes which can break lactose into its monomers and research has shown that most yeasts reported in alcoholic milk fermented products lack genes encoding β -galactosidases, suggesting that they must rely on free monosaccharides or lactic acid (Hittinger et al., 2018). Fructose is found in many food items that could be used as substrates such as marula fruits (*Sclerocarya birrea*), watermelon juice (*Citrus lanatus*), masau fruits (*Ziziphus mauritiana*), to name a few (Dlamini and Dube, 2008; Nyanga et al., 2008; Ogoto et al., 2015). As such, a good strain should be able to utilize fructose and the results below shows that all the strains utilized fructose except for MA2 and MA7. The isolates D4 and T1 were not able to grow on media with maltose while the control strain, D3 and S5 grew very well.

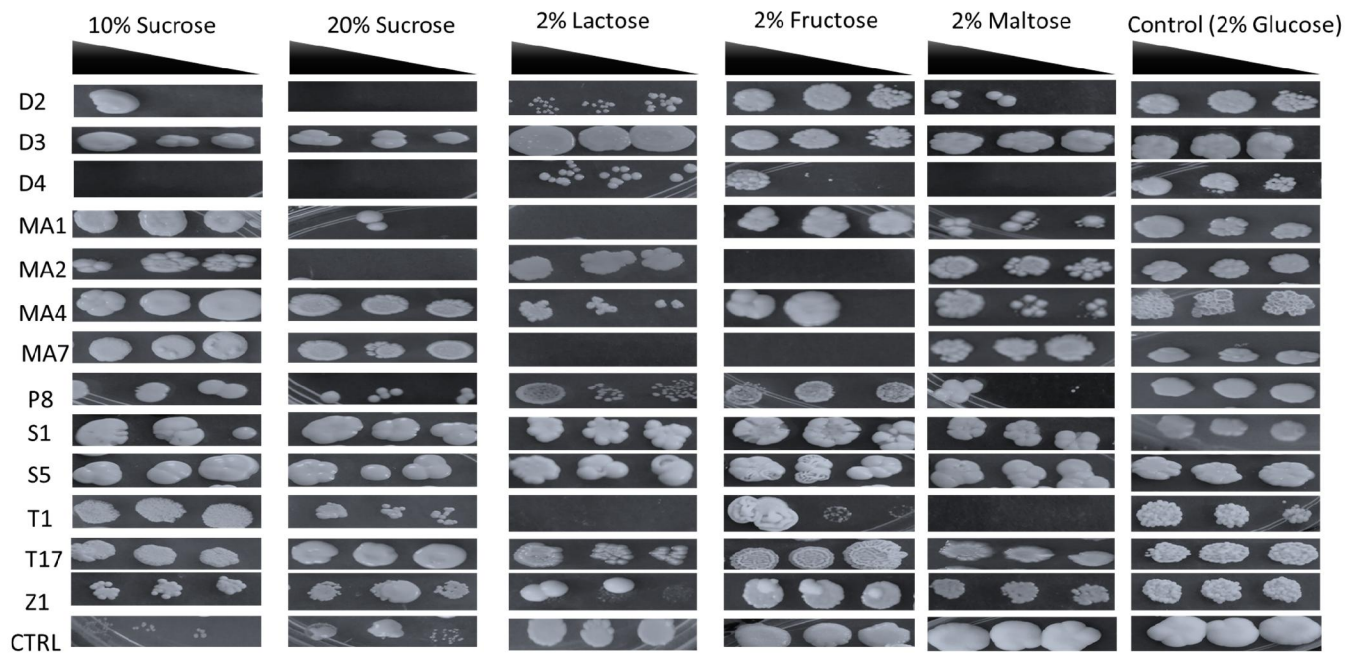


Figure 12: Ability of the isolated yeast strains to utilize different carbon sources. The baker's yeast (*S. cerevisiae*) was used at the control since it is the strain that *khadi* brewers use. The black triangle shows concentrations of the dilutions from left to right (0.5, 0.25 and 0.125).

4.6.3.2 Fermentation capacity

The purpose of this experiment was to determine the amount of CO₂ produced per isolate, with CO₂ production signifying progression of the fermentation process in comparison to the control, *S. cerevisiae* (local commercial baker's yeast). The experiment was done using synthetic media prepared using *G. flava* fruits as a source of nutrients and brown sugar as a carbon source mimicking the *khadi* brewing process. Our results showed that *C. pallidicorallinum* performed exceptionally well followed by *A. leucospermi*, *N. diffluens* and *C. sake* while strains, *A. melanogenum* and *S. cerevisiae* had a long lag phase (Figure 13). The fermentative ability of some of these yeasts, such as *C. sake* and *S. cerevisiae*, was not surprising as they have been

reported in many traditional beverages such as *tej* of Ethiopia (Bahiru et al., 2006), palm wine of Nigeria (Amoa-Awua et al., 2007; Stringini et al., 2009; Tapsoba et al., 2015), *mukumbi* and *kachasu* of Zimbabwe (Mpofu et al., 2008; Nyanga et al., 2013b, 2007; Okagbue and Siwela, 2002).

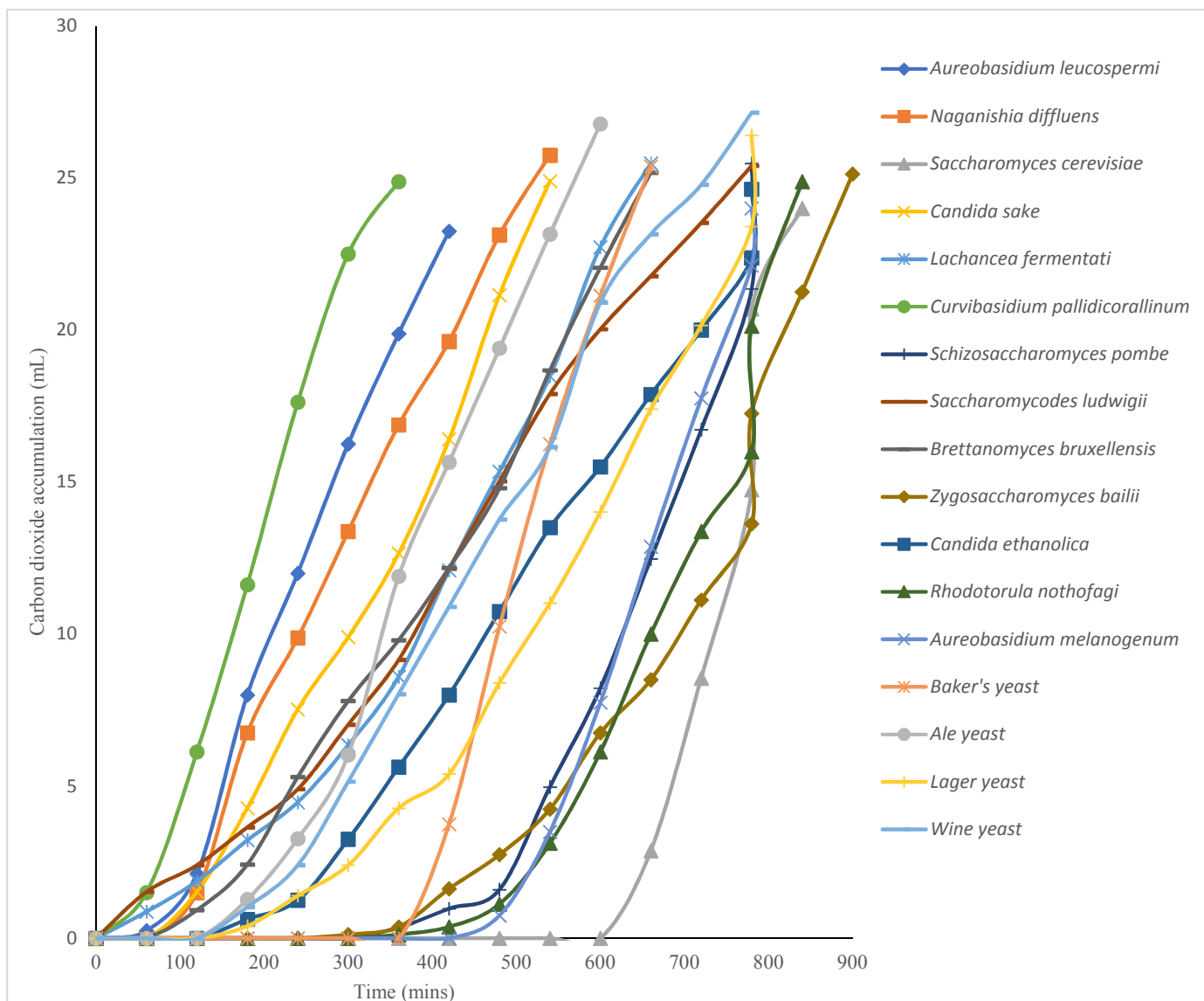


Figure 13: Fermentative capacity of yeast isolates. The graph shows the carbon dioxide produced by the yeast isolates at an interval of 60 minutes. This experiment was performed three times with two replicates each.

CO₂ production rate which directly translates to fermentation rate is an additional critical attribute of yeast isolates (Donalies et al., 2008; Johnson and Echavarri-Erasun, 2011). As such, we investigated the CO₂ production rate. The results below (Figure 14) show that the control (*S. cerevisiae*) performed better than all the test strains isolated from *khadi*. The CO₂ production rate of *L. fermentati* (0.076 ± 0.004 mL/min) was higher than all the 4 controls. Our results suggest that there was a significant statistical difference between all samples when each of them was compared to the commercial baker's yeast (t-test; $p < 0.001$), except for *C. sake*. The *C. sake* could be one of the ideal strains to use for the fermentation of *khadi* but a valid conclusion will be drawn once the strain chemical analysis of its fermentation broth has been done.

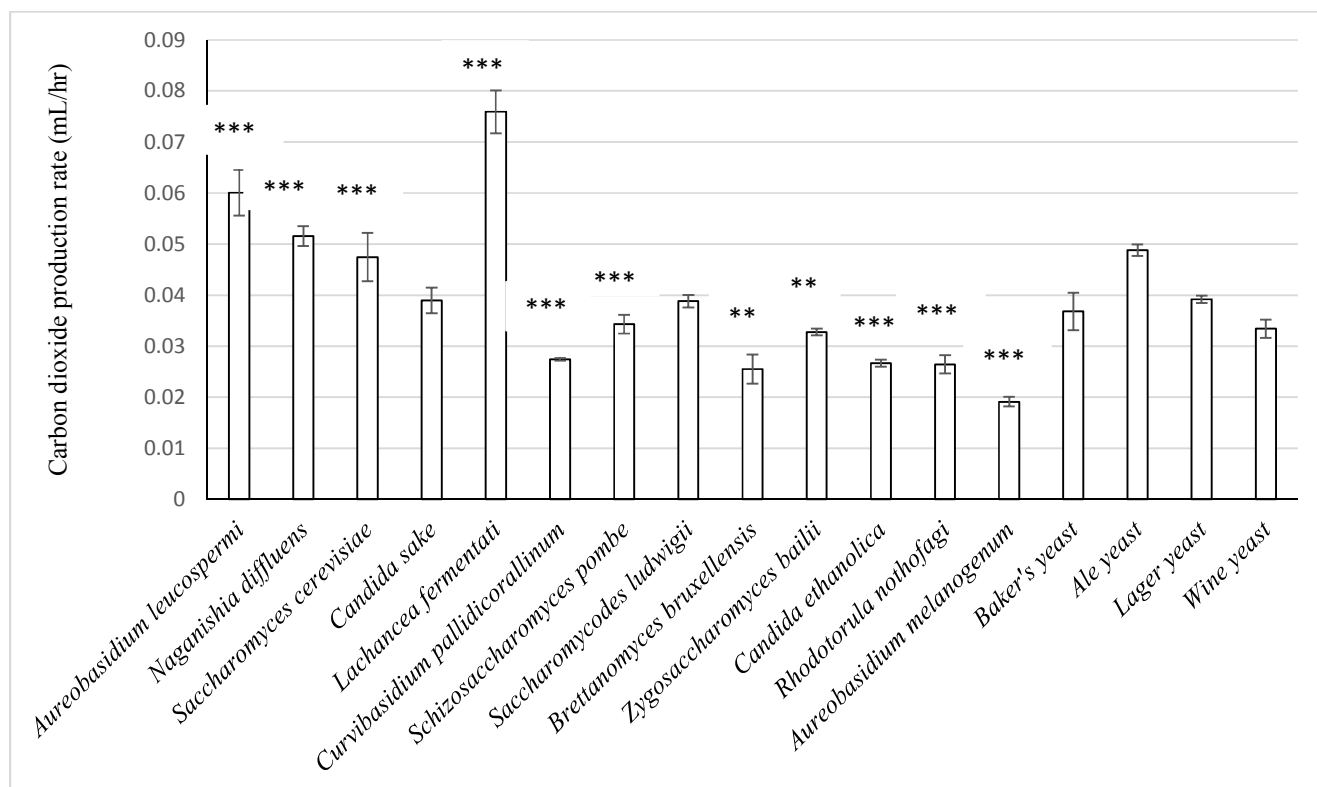


Figure 14: Carbon dioxide production rate of yeast isolates. The average carbon dioxide production rate (mL/hr) of the yeast isolates with the controls (ale, baker's, lager and wine yeasts). The t-test results (isolated yeasts vs baker's yeast) are presented as with asterisks where significance is a * ($P < 0.05$), high significance is ** ($P < 0.01$) and great significance is *** ($P < 0.001$).

4.6.3.3 Ethanol stress tolerance

Ethanol is one of the metabolites produced during *khadi* fermentation. As such, the fermentation process requires a strain that can withstand elevated ethanol concentrations without its growth being inhibited or impaired. The amount of ethanol produced depends on many attributes such as the fermenting microorganism, the amount and types of sugars used as a substrate, and the conditions at which fermentation is carried out, to list a few. Utilization of an organism with

higher resistance to ethanol is important for an efficient fermentation process. All potential isolates were tested for growth on YPD supplemented with different concentrations of ethanol (Figure 15). All isolates were resistant to 3 % ethanol and exhibited growth comparable to when grown in the absence of ethanol except MA2. This ethanol concentration was chosen because ethanol concentration of *khadi* is 3.69 % (v/v) as previously reported by Mapitse et al., (2014). However, as observed in this work, ethanol concentrations in different *khadi* were on average, 5.27 ± 2.02 % (Figure 8). All isolates were resistant to this amount of ethanol except Z1 and MA2. It was noted that ethanol concentrations in different *khadi* could be higher up to about 9% (Figure 8). All the isolates remained resistant to 9 % ethanol except, MA2, T1, and Z1 suggesting that all the resistant isolates have potential to be used as starter cultures to produce higher amounts of ethanol without a decrease in productivity.

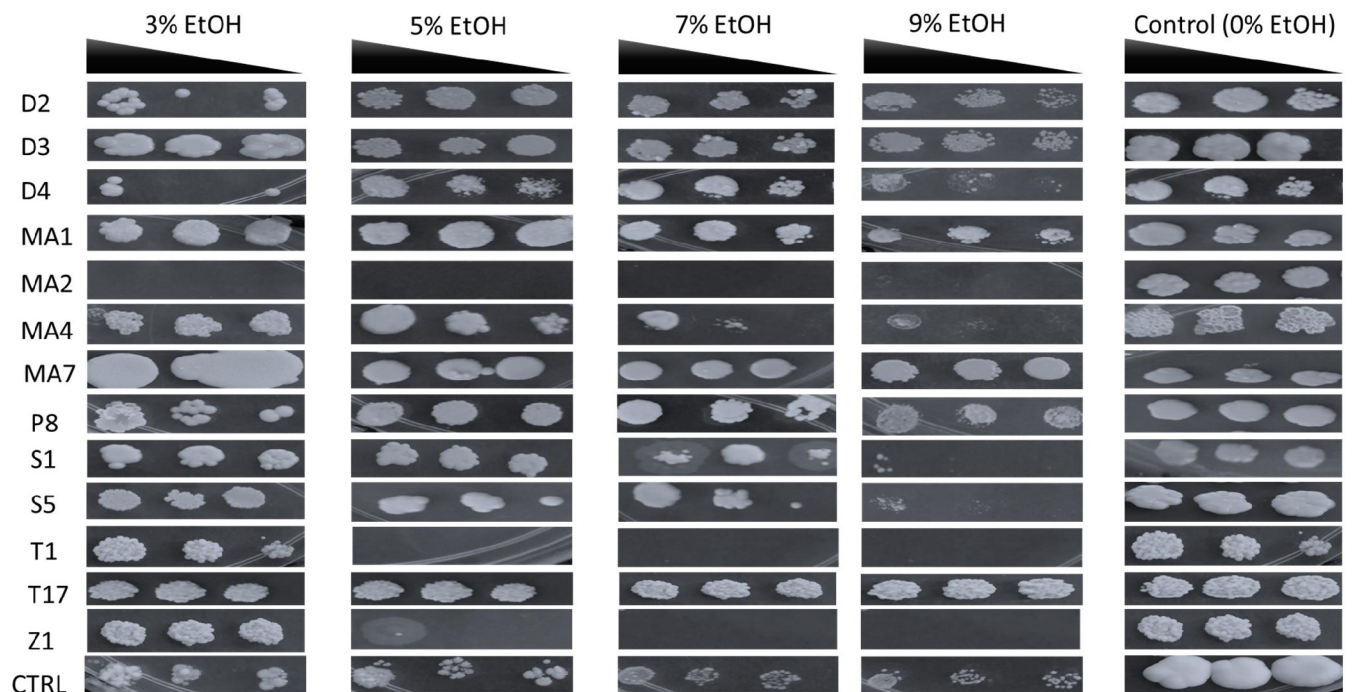


Figure 15: Growth inhibition of the yeast strains due to different ethanol concentrations.

The baker's yeast (*S. cerevisiae*) was used at the control since it is the strain that *khadi* brewers use. The black triangle shows concentrations of the dilutions from left to right (0.5, 0.25 and 0.125).

4.6.3.4 Osmotolerance

Sugar content of fruits is subject to change every season (Yahia and Kader, 2011). The change could bring about high sugar concentrations, which are associated with a higher osmotic stress. Osmotic stress reduces productivity of production strains. Utilization of osmotolerant isolates is an important attribute in brewing. All isolates were tested for osmotolerance by growing them in media supplemented with an osmolyte, in this case NaCl was chosen. The yeast isolates grew on media with 0.5 M NaCl except for MA2 (Figure 16). Their osmotolerance was comparably to the control yeast. However, increasing the osmotic pressure by growing isolates on 1.0 M NaCl was inhibitory for growth of isolates except 5 isolates (D3, D4, MA4 and S1) and the control strain. These results suggest that strains will be highly impaired or inhibited by media with high sugar concentrations. It is noteworthy that S1 was the only strain to grow on 1.5M NaCl although there was no growth on 2.0M NaCl suggesting this isolate as the only isolate of choice in the presence of higher amounts of sugars.

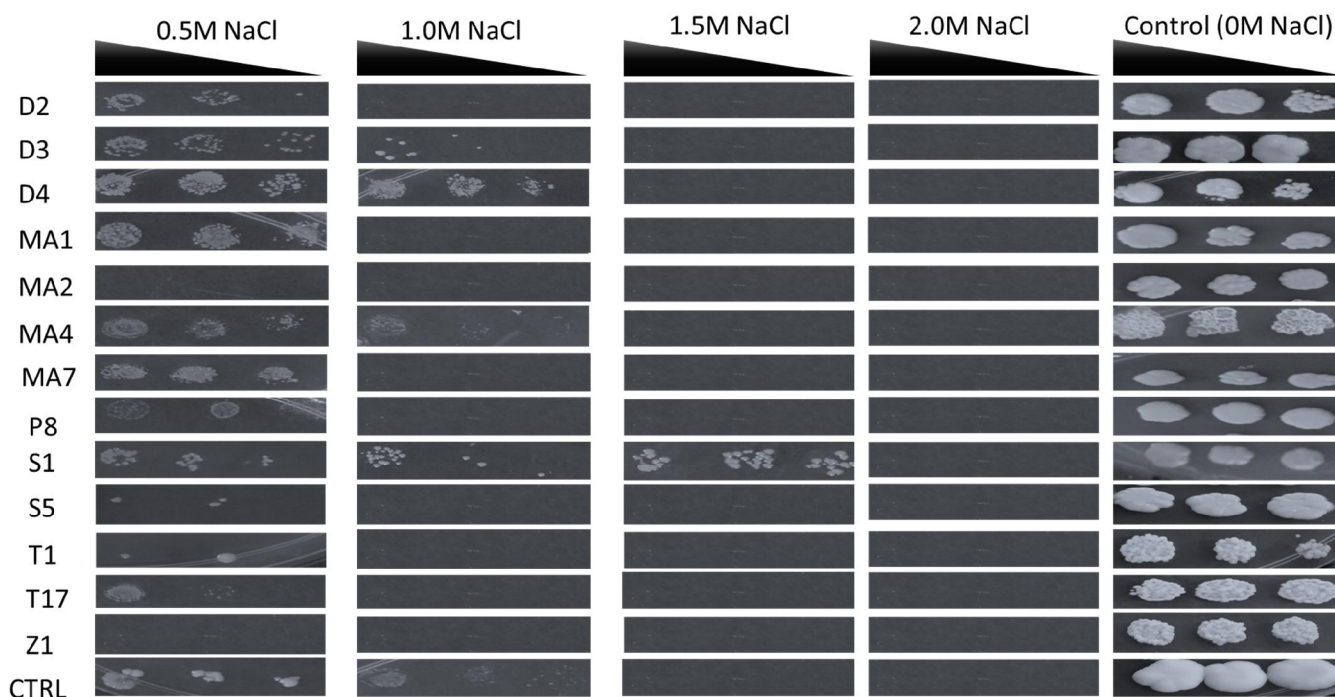


Figure 16: Osmo-tolerance of the yeast isolates due to supplementation with various NaCl concentrations. The baker's yeast (*S. cerevisiae*) was used at the control since it is the strain that *khadi* brewers use. The black triangle shows concentrations of the dilutions from left to right (0.5, 0.25 and 0.125).

4.6.3.5 pH stresses

During the production of *khadi* or any alcoholic beverage, a variety of organic acids are produced which lowers the pH of the alcoholic beverage. From this work, it was found out that *Khadi* is an acidic alcoholic beverage, which was in agreement to previously reports by (Mapitse et al., 2014). These results prompted an investigation into the performance of isolates subjected to various acidic pHs. In addition, it was important to investigate the lowest possible pH at which isolated strains could withstand because it would not be ideal to select a potential isolate that will

be inhibited at a lower pH during the course of fermentation. The results show that all the strains could not withstand pH 1 (Figure 17) as expected. Although some isolates performed well at pH 2, this pH is not ideal for any alcoholic beverage. It should be noted that the average pH recorded among *khadi* in this work was 3.1 ± 0.31 (Figure 8) in agreement to a range from 2.87 to 3.16 reported by Mapitse et al., 2014. All strains grew on media at pH 3 except T1 and Z1. This suggests that most of these isolates could be ideal to withstand low acidic conditions during the *khadi* fermentation. The acidity of alcoholic beverages is important for inhibition of growth of spoilage microorganisms (Manel et al., 2011; Schifferdecker et al., 2014; Swiegers et al., 2009).

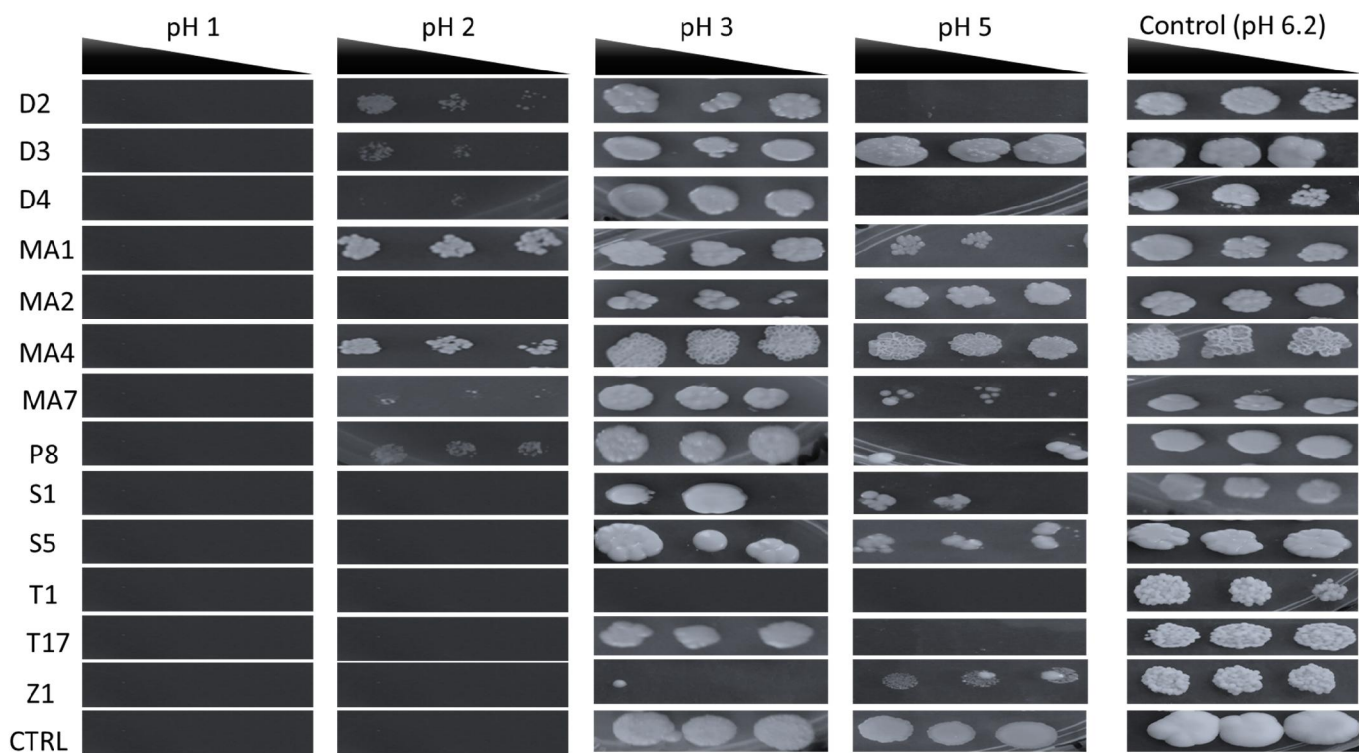


Figure 17: The pH stress tolerance of the yeast isolates. The baker's yeast (*S. cerevisiae*) was used at the control since it is the strain that *khadi* brewers use. The black triangle shows concentrations of the dilutions from left to right (0.5, 0.25 and 0.125).

4.6.3.6 Thermotolerance

Khadi is produced under non-standardized conditions in which fluctuation of temperatures could reduce fermentative capacity. This is because fermentation is an exergonic process and thus there is increase in temperature of the fermentation broth as fermentation proceeds. Therefore, uncontrolled fermentation of *khadi* requires the use of a thermotolerant isolate should there be excessive production of heat during fermentation. In addition, when *khadi* fermentation is done under controlled fermenters, this thermotolerant attribute could be very economic as the costs of cooling the fermentation vessels will be reduced or negated. Therefore, the upper threshold temperatures that the yeast isolates could withstand were investigated. The results suggest that most of the strains performed well at 37 °C and 40 °C except for isolates T1 and Z1 (Figure 18). Higher temperatures were inhibitory for most of the isolates except 5 (D2, D3, D4, MA4, P8). The control yeast was also able to grow at temperature of 43 °C. These results suggest that the isolates have an immense potential to be used for *khadi* production at higher temperatures. The isolates *Curvibasidium pallicorallinum* (MA7), *C. sake* (MA2), *L. fermentati* (MA4), *Saccharomycodes ludwigii* (S1) and *C. ethanolica* (T17) have potential for production of *khadi*.

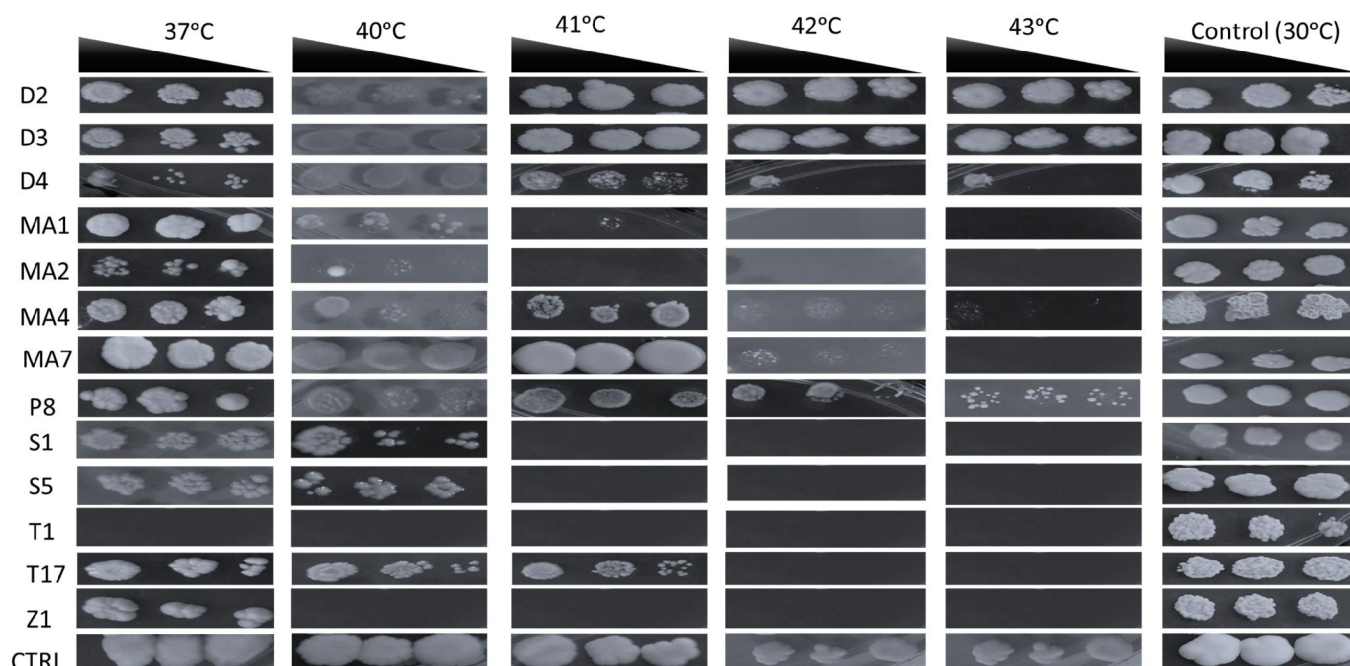


Figure 18: The thermo-tolerance of the isolates. The baker's yeast (*S. cerevisiae*) was used at the control since it is the strain that *khadi* brewers use. The black triangle shows concentrations of the dilutions from left to right (0.5, 0.25 and 0.125).

4.7 Safety in bioprocessing of *khadi*

4.7.1 Identification of molds contaminants of *khadi*

To check for molds contamination, the *khadi* samples were grown on molds isolation media. The results showed that the majority of the samples were contaminated with phylogenetically diverse molds species (Figure 19). A total of 14 molds isolates were identified, some of which include *Aspergillus ochraceus*, *Cladosporium sphaerospermum*, *Cladosporium cladosporioides*, *Phoma eupyrena* and *Setosphaeria turcica*, to list a few (Table 13 and Figure 19). The predominant isolates were *C. sphaerospermum* (Serowe and Maun), *A. ochraceus* (Palapye and Tonota) and

Flavodon ambrosius (Mmashoro) (Table 13), each attributing 21.4 % of the total isolates. The introduction of molds to *khadi* might be through the fruits or as a contamination from the brewers or brewing utensils. Visible molds growth on *khadi* may reduce the shelf life of the product and make it not fit for human consumption thus meaning molds are undesirables in foods.

Molds isolates such as *Aspergillus ochraceus* in beverages can be an indicator of the possible presence of mycotoxins. However, molds in most cases does not imply the presence of mycotoxins. Sometimes it means the food item has necessary ideal conditions such as high water activity, the presence of sugars, and the presence of organic acids capable of reducing pH (Snowdon and Cliver, 1996) hence a possible explanation for the presence of these molds isolates on the *khadi* samples. *Aspergillus ochraceus* is of great concern as it has been previously reported to produce *aflatoxins* and *ochratoxins* (Kharayat and Singh, 2018) which are carcinogenic, nephrotoxic, hepatotoxic and teratogenic to mammalian cells (Roseanu et al., 2010). On the other hand, *S. turcica* is a hemi-biotrophic plant pathogen (parasitic on living and dead tissue) and heterothallic (sexes that reside in different individuals) whose usual host is maize and sorghum. Due to its hemi-biotrophic ability, hypothetically *S. turcica* could be passed from *G. flava* fruits to the *khadi*. The other isolates of interest are *C. sphaerospermum* and *C. cladosporioides*, which are filamentous molds previously isolated from flowers of edible fruit plants as well as being isolated from honey, dairy products and grapes prior to harvesting (Cosme et al., 2018; Hill, 2015; Moubasher et al., 2018; Snowdon and Cliver, 1996). The *Cladosporium* species have been previously reported to be mycotoxin secreting and potentially pathogenic fungus frequently occurring in outdoor environments (Hadi, 2017). With that said,

the presence of all these molds could be an indicator for mycotoxin presence in the *khadi* samples.

Table 13: Identity of molds isolates from the *khadi* samples based on D1/D2 domains of 26S rDNA.

Species name	Collection number	Accession number	Identity %
<i>Aspergillus ochraceus</i> (2)	Tonota 2	KX958037.1	99
<i>Aspergillus ochraceus</i>	Palapye 1	KX958037.1	99
<i>Chaetomium longiciliata</i>	Maun 3	KP336823.1	99
<i>Cladosporium cladosporioides</i>	Palapye 1	KM246047.1	99
<i>Cladosporium sphaerospermum</i>	Serowe 1	KY873376.1	99
<i>Cladosporium sphaerospermum</i> (2)	Maun 3	KY873376.1	99
<i>Flavodon ambrosius</i> (3)	Mmashoro 3	NG_060421.1	99
<i>Periconia thailandica</i>	Serowe 2	KY753888.1	99
<i>Phoma eupyrena</i>	Palapye 3	JF766684.1	99
<i>Setosphaeria turcica</i>	Maun 1	LT715640.1	99

Note: The number in the bracket shows strains with the same accession number from the same location.

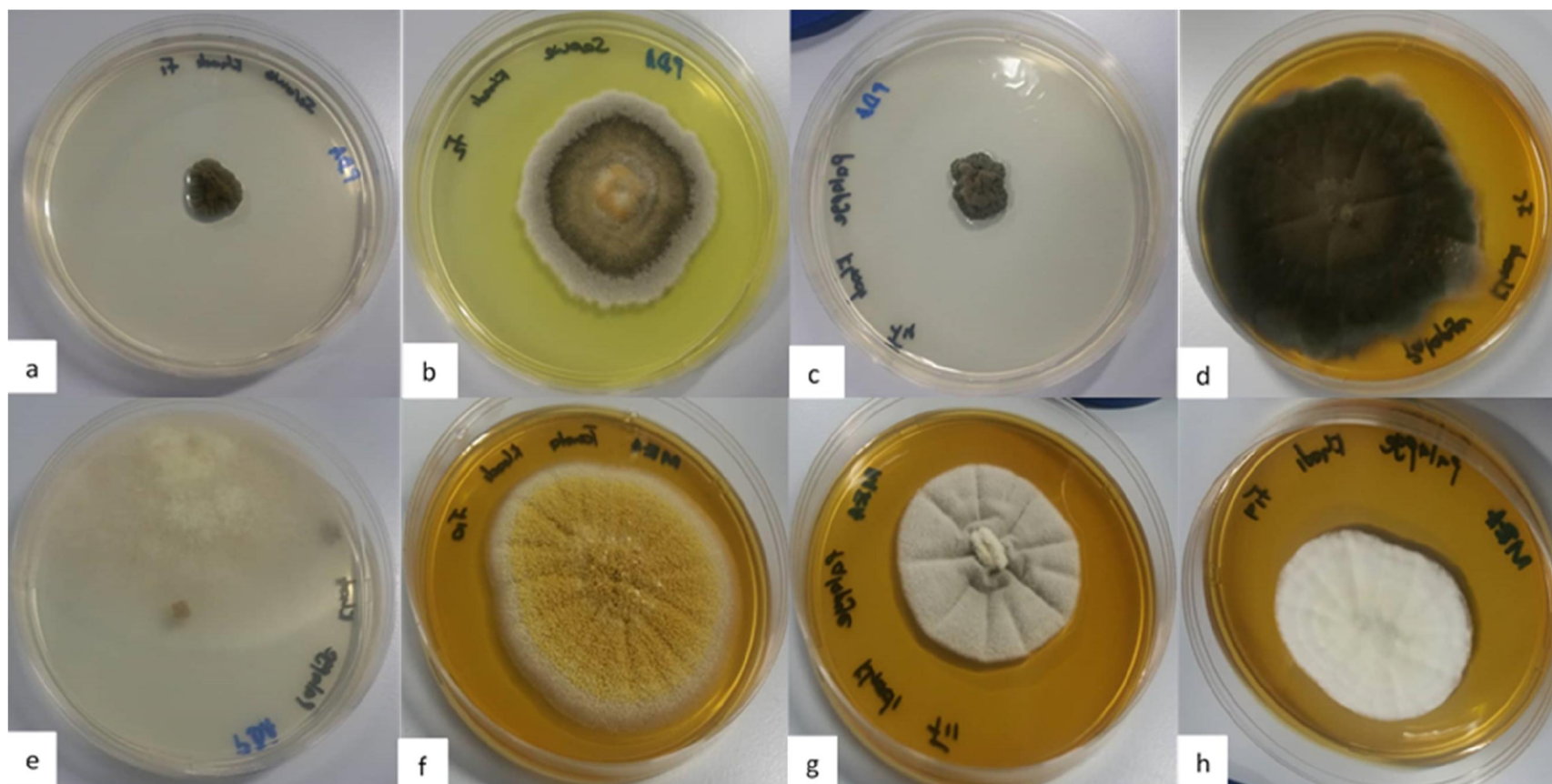


Figure 19: A picture of the molds isolates from *khadi* samples namely: (a) *Cladosporium sphaerospermum*, (b) *Periconia thailandica*, (c) *Cladosporium cladosporioides*, (d) *Setosphaeria turcica* (e) *Flavodon ambrosius* (f) *Aspergillus ochraceus*, (g) *Phoma eupyrena* and (h) *Chaetomium longiciliata*.

4.7.2 Mycotoxins quantification: Evidence Investigator calibration

The presence of molds isolates (Figure 19) in the *khadi* samples prompted us to analyze this brew for mycotoxins. Mycotoxin quantification was carried out using the Randox Evidence Investigator. To optimize the sensitivity of the method and test its accuracy, the Randox Evidence Investigator was calibrated using the provided calibrators of a known concentration. The acquired data was then used to provide information of the limits of detection (LODs) and limits of quantification (LOQs). LOD and LOQ are terms used to describe the smallest concentration of an analyte that can be reliably measured by an analytical procedure with an acceptable degree of accuracy and precision. The LOD and LOQ were calculated based on threefold and tenfold signal to noise (S/N) ratios, respectively (Table 14). The LOD ranged from 0.13 to 101.33 while the LOQ ranged from 0.43 to 337.78 with DON having the highest LOD and LOQ. The linearity was established by the calibration curve based on the expected values and actual values from calibrators. The coefficient correlations R^2 ranged from 0.97 to 0.99 thus showing good linearity (Table 14) hence method validation.

Table 14: The calibration results from the Radox Evidence Investigator.

Compound	Linear equation	R ²	SD	LOD	LOQ
PAX	Y = 0.9634x + 0.0252	0.99	6.48	20.18	69.39
FUM	Y = 1.0061x - 0.1004	0.97	0.68	2.03	6.77
OTA	Y = 1.0088x - 0.0131	0.98	0.27	0.81	2.70
AFG1	Y = 0.9883x - 0.0103	0.98	0.33	1.01	3.38
EA	Y = 1.0056x - 0.1973	0.97	4.49	13.41	44.70
DAS	Y = 1.0816x - 0.4864	0.97	7.33	20.33	67.76
DON	Y = 1.0907x - 2.4123	0.98	36.84	101.34	337.78
T2	Y = 1.0935x - 0.0721	0.98	1.47	4.04	13.47
AFB1	Y = 1.0769x - 0.0023	0.98	0.05	0.13	0.43
ZEA	Y = 1.0766x - 0.0459	0.99	0.72	2.02	6.73

Notes: PAX = paxilline, FUM = fumonisin, OTA = ochratoxin A, AFG1/G2 = aflatoxin G1/G2, ERG = ergot alkaloids, DAS = diacetoxyscirpenol, DON = deoxynivalenol, AFB1/B2 = aflatoxin B1/B2, ZEA = zearalenone.

Three samples from three different sampling areas were then spiked with a known concentration of AFB1, FB1 and OTA to test the efficiency of the acetonitrile method and the Myco-10 method (according to the manufacturer's instruction) (Table 15). The recovery of the samples ranged from 12.05 % to 107.3 % using the acetonitrile method where the best recovery was observed on FB1 and AFB1. The acetonitrile method failed to extract OTA from all the three samples as the spike recovery was very low. The low recovery of mycotoxins OTA could possibly mean the acetonitrile extraction method was not optimal for these mycotoxins and this could be affecting the extraction of these toxins from the *khadi* samples thus giving inaccurate

results. Table 14 shows that the challenge is not with the biochip as the quantification of the all control standards read values close to the true concentrations as stated by the manual provided by the manufacturer. This is further confirmed by the R^2 of the sample calibrators. The results in Table 15 also show that the Myco-10 extraction method was not suitable for use in this experiment. The Myco-10 extraction method was based on the use of chemicals namely acetonitrile, methanol and water as the extraction solvents and no organic salts. The acetonitrile method was probably more effective as it countered the presence of water in *khadi* through the use of organic salts to bind to the water molecules (Font et al., 2017). This step was not there in the Myco-10 extraction method. This method did not yield meaningful results and thus it was deemed unsuitable for use in this work.

Table 15: Comparison of the product recovery from the spiked samples using the acetonitrile method and the Myco-10 method.

Sample	FB1 (1 ppm)	OTA (2 ppm)	AFB1 (0.1 ppm)
Maun 1 (ACN method)	91.40 %	18.80 %	53.10 %
Mmashoro 2 (ACN method)	103.70 %	12.05 %	45.80 %
Serowe 1 (ACN method)	107.30 %	23.50 %	61.90 %
Serowe 1 (Myco-10 method)	-98.2 %	-5.11 %	-75 %

4.7.3 Mycotoxins from *khadi*

After the optimization of the acetonitrile method, the *khadi* samples were analysed for presence of mycotoxins. Table 16 shows the mycotoxins from the *khadi* samples. All the samples had fumonisin, ochratoxin A, diacetoxyscirpenol and zearalenone. The least detected toxins from the

brews were paxilline (detected from Mmashoro 1, Mmashoro 2, Palapye 1, Palapye 3, Serowe 1 and Serowe 3), deoxynivalenol (detected from Maun 1, Maun 2, Serowe 1, Tonota 1, Tonota 2 and Tonota 3) and aflatoxin B1/B2 (detected from Mmashoro 3, Serowe 1 and Tonota 2). The Serowe 1 sample was observed to have all the mycotoxins detected from it. The overall average quantification shows that the highest detected mycotoxin was DAS (22.9 ± 8.12 ppb) and the least detected toxin was aflatoxin B1/B2 (0.0023 ± 0.0023 ppb). The average of the other toxins being PAX (0.56 ± 0.47), FUM (1.27 ± 0.55), OTA (2.11 ± 0.92), AFG1/G2 (0.088 ± 0.02), ERG (3.9 ± 0.68), DON (11.1 ± 5.81) and ZEA (0.13 ± 0.1).

Mycotoxins such as zearalenone have been detected from traditional opaque beers of Botswana (Nkwe et al., 2005) while others such as ochratoxin A, deoxynivalenol, fumonisin B1 and fumonisin B2 have been detected in many European beers (Bertuzzi et al., 2011). In another study on the maize-based alcoholic beverage of South Africa, *umqombothi*, the authors detected mycotoxins such as T2, DON, fumonisin, zearalenone and aflatoxins from the beverage with evidence pointing at the raw material or substrate as the source of the mycotoxins (Adekoya et al., 2018; Peters et al., 2017). Dried fruits have more mycotoxins than fresh fruits. Mycotoxins accumulate during the harvesting, drying and storage due to conditions that favour mycotoxin accumulation (Drusch and Ragab, 2003). The *A. ochraceus* produces ochratoxin A, which was detected in all the *khadi* samples in this work. The detection of other mycotoxins such as aflatoxins is characteristic of other molds such as *A. flavus*, *A. parasiticus*, *A. bombycis*, *A. ochraceus*, *A. nomius*, and *A. pseudotamar* (Bircan, 2009; Kharayat and Singh, 2018; Kumari et al., 2015; Lyumugabe et al., 2012; Roseanu et al., 2010; Zain, 2011). In this work, only *A. ochraceus* was isolated from *khadi* and thus this is the only possible source of aflatoxins

detected. Due to the limitation of the rapid analysis method and acetonitrile extraction method, no comparison was made to the FDA or EC law on the mycotoxin maximum allowed limits.

Table 16: The quantification of mycotoxins from *khadi* samples in ppb.

Sample	PAX	FUM	OTA	AFG1/G2	ERG	DAS	DON	T2	AFB1/B2	ZEA
Control	1.51	0.27	0.057	0.066	1.10	1.30	8.89	0.25	0.0081	0.14
Maun 1	0	1.30	2.53	0.092	4.57	27.06	5.57	0.24	0	0.16
Maun 2	0	1.48	2.26	0.1116	4.86	25.13	10.02	0.30	0	0.094
Maun 3	0	0.61	0.87	0.067	2.43	14.53	0	0.06	0	0.091
Mmashoro 1	0.23	1.14	1.97	0.12	4.16	21.12	0	0.20	0	0.11
Mmashoro 2	0.51	1.46	2.26	0.088	4.45	24.74	0	0.15	0	0.12
Mmashoro 3	0	1.90	2.79	0.096	4.10	29.23	0	0.079	0.0006	0.13
Palapye 1	0.27	1.55	2.22	0.060	3.60	25.21	0	0.13	0	0.19
Palapye 2	0	1.81	2.33	0.079	3.26	27.30	0	0.13	0	0.12
Palapye 3	0.33	2.27	3.52	0.072	3.78	29.91	0	0.093	0	0.068
Serowe 1	1.32	1.14	2.13	0.12	3.22	26.06	21.86	0.33	0.0049	0.49
Serowe 2	0	1.45	1.89	0.058	3.80	23.11	0	0.091	0	0.13
Serowe 3	0.16	1.15	1.76	0.074	3.35	22.19	0	0.12	0	0.11
Tonota 1	0	1.72	3.84	0.11	4.61	33.79	8.46	0.24	0	0.18
Tonota 2	0	1.38	2.82	0.12	4.93	29.54	10.95	0.26	0.0013	0.19
Tonota 3	0	1.26	2.58	0.078	3.84	26.51	7.09	0.27	0	0.13
Letlhakane 1	0	1.02	1.60	0.07	3.56	19.63	0	0.027	0	0.042
Letlhakane 2	0	0.18	0.44	0	0	4.34	0	0	0	0.009
Letlhakane 3	0	0.14	0.33	0	0	3.47	0	0	0	0.004

Notes: PAX = paxilline, FUM = fumonisin, OTA = ochratoxin A, AFG1/G2 = aflatoxin G1/G2, ERG = ergot alkaloids, DAS = diacetoxyscirpenol, DON = deoxynivalenol, AFB1/B2 = aflatoxin B1/B2, ZEA = zearalenone.

Chapter 5: General discussion and conclusion

5.1 Summary of study investigations and findings

Khadi is a homemade traditional beverage, produced from the spontaneous fermentation of *G. flava* fruits, whose fermentation is carried out by unknown microbiota. *Khadi* fermentation process varies based on the preference of the brewers. As such, there is no standard procedure of producing this beverage. Therefore, there are a lot of product inconsistencies, which have led to negative health implications. Thus, this work sought to document the step-by-step process of brewing *khadi* using this traditional beverage and its substrate as a source of fermentative microbes. This was done by sequencing 16S rDNA for bacterial identification and 26S rDNA for yeasts identification.

The source of these microorganisms could be the *G. flava* fruits and the fermentation equipment used. The isolates identified from the *G. flava* fruits were not limited to yeasts only, we also identified some bacteria. The *Bacillus* spp. were predominant species in the *G. flava* fruits. The dominance of *Bacillus* species was not surprising as they are ubiquitous microbes that are common in soil, air, dust, water as well as dried and processed foods of plant origin.

The microbial diversity of *khadi* was mostly made up of phylogenetically diverse consortium of yeast and molds. Interestingly, no bacteria survived the fermentation process. The yeast diversity of *khadi* showed that this beverage was produced by a mixed culture of yeast. The consortium identified from *khadi* was made up of both conventional yeasts and non-conventional yeasts. Some of the identified isolates are spoilage yeasts such as *B. bruxellensis* and *Z. bailii* (Barata et al., 2012; Chatonnet et al., 1995; James and Stratford, 2003; Jeffries et al., 2015; Licker et al., 1998; Schifferdecker et al., 2016). Even though *khadi* is supposed to be produced through spontaneous fermentation, it is noteworthy that our results also showed some of the brewers inoculated using the locally available commercial baker's yeast.

As already stated above, the *khadi* consortium also included some molds. The identified were *Cladosporium sphaerospermum*, *Periconia thailandica*, *Cladosporium cladosporioides*, *Aspergillus ochraceus*, *Phoma eupyrena*, *Setosphaeria turcica*, *Chaetomium longiciliata* and *Flavodon ambrosius*. The predominant molds isolates were *C. sphaerospermum*, *A. ochraceus* and *Flavodon ambrosius*. The detection of these molds could be a possible indication of the presence of mycotoxins thus we quantified mycotoxins from all the *khadi* samples. The mycotoxins quantification results showed that, on average, the highest detected mycotoxin was DAS and the least detected toxin to be aflatoxin B1/B2. Some of the mycotoxins in *khadi* have previously been reported in beers from Africa and Europe, and these include *aflatoxins*, *deoxynivalenol* (DON), *fumonisin*, *ochratoxins*, *ergot alkaloids*, and *zearalenone* (Ezekiel et al., 2015; Fernández-Cruz et al., 2010; Galvis-Sánchez et al., 2008; Inoue et al., 2013; Kirui et al., 2014; Richard, 2007; Zain, 2011).

To select a possible starter culture for *khadi* fermentation, the yeasts were characterized and exposed to many strenuous conditions which included different concentrations of ethanol, sodium chloride, carbon sources, different pH and different temperatures. On average, the results showed that isolates *C. pallicorallinum* (MA7), *C. sake* (MA2), *L. fermentati* (MA4), *Saccharomyces ludwigii* (S1) and *C. ethanolica* (T17) performed well on all these experiments including the fermentation profiling. These are the strains that further research could be conducted in search of a potential starter culture.

5.2 The significance of the findings to food hygiene and safety

Spontaneous and uncontrolled fermentation of *khadi* could lead to many health implications. This fermentation technique leads to the production of undesirable metabolites some of which

could have health implications on the *khadi* consumers. As such, our findings could be used to develop a reliable starter culture which could be sold to *khadi* brewers. *Khadi* is a source of income for many households thus a starter culture could benefit many families whose lives depend on *khadi* sales. Our characterization results showed that the best strains to use for *khadi* fermentation are *C. pallicorallinum*, *C. sake*, *L. fermentati*, *Saccharomyces ludwigii* and *C. ethanolica*. These strains are all non-conventional yeasts whose fermentation processes could lead to the production of unique flavors. With these strains, the people could be able to produce a flavor rich product. Future-work will include the chemical profiling of the beverages produced using yeast isolated from *khadi* to confirm their selection for commercialization.

5.3 The major strengths and weaknesses of study methodology

Documentation of *khadi* preparation methods was based on scientifically accepted methods. The methods used during the enumeration, isolation and identification of bacteria, molds and yeasts from the *G. flava* fruits and *khadi* were scientifically accepted methods. The characterization of bacterial isolates and yeasts were studied using scientifically accepted methods. The methods of genetic characterization of yeasts from *khadi* may need improvement which could be done not only through *in-silico* analysis but also the actual RFLP to confirm the results from the *in-silico* work. There is need to conduct antibiotic susceptibility of the bacterial isolates from *G. flava* fruits after identification by sequencing of 16S rDNA

5.4 Conclusion

This study presents the first process documentation and microbiological characterization of a prominent traditional alcoholic beverage in Botswana. The survey questionnaire revealed that *khadi* is brewed from ripened sun-dried *G. flava* fruits, an inoculum of previously fermented *G. flava* fruits, brown table sugar and warm water only. The survey revealed that *khadi* fermentation employs spontaneous of these ingredients to produce *khadi*. The microbial diversity of *G. flava* fruits and *khadi* showed that there was predominance of non-fermentative Basidiomycetous yeasts and bacteria on the ripened sun-dried *G. flava* fruits while the *khadi* samples harbored fermentative *Saccharomyces* yeasts and non-*Saccharomyces* yeasts as well as molds. The results presented in this work suggest that *khadi* is a product of mixed culture fermentation. It is plausible that local commercially available baker's yeast is regularly used as part of the fermenting starter culture whereas some brewers employed spontaneous fermentation. Evaluation of potential starter cultures suggests that *C. pallicorallinum*, *C. sake*, *L. fermentati*, *Saccharomycodes ludwigii* and *C. ethanolica* isolated from *khadi* are able to withstand various stressful conditions. Since stress resistance is also key to the bioprocess development of *khadi* for commercialization the thesis highlights that these isolates have potential as starter cultures. Further studies to test for the functionality of the mixed consortia could be useful for development of starter cultures for a consistent product quality towards commercialization of *khadi*. Like most naturally fermented beverages, *khadi* was found to contain mycotoxin producers and thus further investigation to quantify mycotoxins was deemed necessary to determine the safety of this alcoholic beverage. The rapid analysis of mycotoxins from *khadi* revealed the presence of mycotoxins such as DAS and DON. These mycotoxins can be harmful

to the *khadi* consumers thus need for the detoxification of the raw materials and the final product before it is consumed.

5.5 Recommendations

To improve this work, yeast isolates from *khadi* could be used to brew *khadi* under controlled and standardized conditions and then subsequent characterization of the product using advanced chromatographic methods with hope to produce a high-quality product. There is need to do a thorough investigation of the presence of other microorganisms through use of culture independent methods such as microbiomics. This is important to reveal other microorganisms that could have been important in improvement of the quality of *khadi*. Chemical profiling of *khadi* to quantify sugars, organic acids, aroma compounds and alcohols should could be instrumental to document different flavors of these different beverages as well as to detect primary or secondary metabolites that could be of harm to the consumers. There is need to evaluate the ethanol production capacity or the contribution of each of the yeast isolates as an important attribute towards development of potential starter cultures. This work did not focus on other sources of yeasts, which could have been responsible for *khadi* fermentation. For example, the drums (vessels) used for brewing could have been the source of the microflora. In the future, the is need to characterize the different and amount of sugars found in *G. flava* in order to understand their contribution to the fermentation process of *khadi*.

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Appendices

Appendix A

Questionnaire

I, Koketso Motlhanka, a Master's student of Botswana International University of Science and Technology, am conducting a research project on the Microbiological and Chemical characterization of *khadi*, a reddish-brown alcoholic beverage made from brown sugar and *Grewia flava* fruits from Botswana. The purpose of the study is to find out the preparation steps and consumption of *khadi* in Botswana. You are requested to assist by answering the following questionnaire. Your participation will be highly appreciated.

Please tick (✓) in the boxes as appropriate.

Location.....

Sex Male ☐ Female ☐

Age

19-25 ☐

26- 35 ☐

36-45 ☐

45 and older ☐

Occupation:

Unemployed ☐

Self-employed ☐

Retired ☐

Farmer ☐

Civil servant ☐

Please assist by answering the following questions.

- 1) What ingredients do you use to prepare *Khadi*?

.....
.....

- 2) Do you add starter culture or inoculum when preparing *khadi*?

Yes ☐

No ☐

- 3) If yes to the above question, what is the source of your inoculum?

.....
.....

- 4) If no to the above question, what do use as a starter culture of your fermentation?

.....
.....

- 5) How much starter culture do you add to initiate *khadi* fermentation?

Half a cup ☐

One full cup ☐

One and half cups ☐

Two cups ☐

If more specify.....

- 6) At which temperature do you incubate your *khadi* to ferment?

.....
.....

- 7) How long does your *khadi* take to ferment?

6 hours ☐

12 hours ☐

18 hours ☐

24 hours ☐

36 hours ☐

48 hours ☐

- 8) How do you know fermentation of the *khadi* is complete?

Production of bubbles ☐

Taste ☐

Production of foam ☐

Depletion of ingredients ☐

Other, specify.....

- 9) What type of vessel do you use to ferment your *khadi*?

Plastic drum ☐

Clay pot ☐

Calabash ☐

Other, specify.....

- 10) What temperature do you store the *khadi* ingredients (especially *Grewia flava* fruits)?

.....
.....

- 11) How do you store the *Grewia flava* fruits?

.....
.....

Appendix B

Table B1: The yeast enumeration results in log₁₀CFU/mL from the *khadi*.

Sample	Experiment 1			Experiment 2			Experiment 3			Average	Standard deviation
Letlhakane 1	5.827	5.226	4.462	5.822	5.474	4.315	5.878	5.561	5.257	5.314	0.576
Letlhakane 2	5.948	5.374	4.609	5.986	5.419	4.447	6.030	5.399	4.452	5.296	0.650
Letlhakane 3	5.608	4.944	4.079	5.602	4.861	4.125	5.479	4.905	4.014	4.846	0.649
Maun 1	6.090	6.205	6.544	6.181	5.785	6.672	6.167	6.259	6.843	6.305	0.325
Maun 2	5.454	5.630	5.523	5.454	5.732	5.802	5.466	5.817	6.014	5.655	0.199
Maun 3	5.553	6.156	6.156	5.645	6.276	6.091	5.677	6.225	6.166	5.994	0.283
Mmashoro 1	5.995	5.353	4.294	5.930	5.312	4.315	6.035	5.260	4.426	5.213	0.716
Mmashoro 2	6.047	4.808	3.602	5.882	5.361	4.519	5.899	5.347	4.690	5.128	0.799
Mmashoro 3	5.633	5.137	4.336	5.437	5.154	4.301	5.540	5.170	4.519	5.025	0.514
Palapye 1	6.185	6.689	6.956	6.309	6.365	6.811	6.265	6.640	6.847	6.563	0.286
Palapye 2	6.327	6.540	6.751	6.269	6.560	6.568	6.286	6.598	6.693	6.510	0.176
Palapye 3	5.954	6.144	6.380	6.057	6.230	6.114	6.066	6.420	6.054	6.158	0.157
Serowe 1	5.780	5.877	5.426	5.773	5.756	6.472	5.750	5.748	5.222	5.756	0.340
Serowe 2	6.335	5.989	5.268	6.272	5.941	5.281	6.271	5.957	5.279	5.844	0.450
Serowe 3	5.987	5.346	4.771	5.848	5.377	4.748	5.922	5.359	4.687	5.338	0.514
Tonota 1	4.519	4.301	4.301	4.374	4.000	4.000	4.491	4.477	4.824	4.365	0.259
Tonota 2	6.029	6.242	6.487	6.023	6.365	6.564	6.022	6.246	6.431	6.268	0.209
Tonota 3	5.929	6.219	6.447	5.985	6.294	6.409	6.073	6.221	6.431	6.223	0.193

Appendix C

Table C1. *S. cerevisiae* yeast strains PCR-RFLP of 26S region of the rRNA using three restriction endonucleases (*HaeIII*, *HinfI*, *GluI* and *RsaI*).

Isolate name	Location designation	Restriction patterns				
		AP ^a	<i>HaeIII</i>	<i>HinfI</i>	<i>GluI</i>	<i>RsaI</i>
Commercial bakers' yeast		582	322, 160, 97, 3	209, 179, 147, 47	345, 186, 29, 22	254, 225, 47, 41, 15
Ale yeast, <i>Saccharomyces cerevisiae</i>		555	322, 124, 109	209, 156, 145, 48	331, 186, 38	225, 218, 65, 47
Lager yeast, <i>Saccharomyces pastorianus</i>		556	427, 129	209, 152, 148, 47	336, 186, 34	225, 223, 61, 47
Wine 1, <i>Saccharomyces bayanus</i>		555	322, 124, 109	209, 156, 143, 47	331, 186, 38	225, 218, 65, 47
Wine 2, <i>Saccharomyces cerevisiae</i>		555	322, 124, 109	209, 156, 145, 48	331, 186, 38	225, 218, 65, 47
AC1MIII	Mmashoro 3		322, 134, 122	209, 179, 147, 47	329, 186, 63	225, 205, 90, 47, 11
AC2MIII	Mmashoro 3	584	237, 163, 99, 85	264, 193, 127	370, 186, 28	584
AC3MIII	Mmashoro 3	584	322, 162, 72, 28	209, 181, 147, 47	345, 186, 24, 17, 12	256, 225, 56, 47
AC5MIII	Mmashoro 3	587	322, 161, 81, 13, 10	209, 180, 151, 47	248, 219, 97, 23	255, 225, 60, 47
BC1MII	Mmashoro 2	586	322, 161, 103	209, 180, 150, 47	345, 186, 32, 23	284, 255, 47
BC4MII	Mmashoro 2	585	322, 161, 102	209, 180, 149, 47	345, 186, 31, 23	255, 225, 58, 47
L3	Letlhakane 1	580	322, 165, 98	209, 179, 145, 47	345, 186, 27, 22	279, 245, 47

L6	Letlhakane 2	587	322, 165, 100	409, 184, 147, 47	345, 186, 29, 27	259, 225, 56, 47
L7	Letlhakane 2	588	322, 166, 100	209, 185, 147, 47	345, 186, 29, 28	260, 225, 56, 47
L8	Letlhakane 2	587	322, 165, 100	209, 184, 147, 47	345, 186, 29, 27	259, 225, 47, 41, 15
L10	Letlhakane 3	587	322, 165, 100	209, 184, 147, 47	345, 186, 29, 27	259, 225, 47, 41, 15
L11	Letlhakane 3	591	322, 169, 100	209, 188, 147, 47	346, 186, 30, 29	225, 135, 128, 47, 40, 16
M1	Maun 1	588	322, 161, 101	209, 185, 147, 47	345, 186, 29, 28	260, 225, 47, 41, 15
M6	Maun 2	581	322, 161, 98	209, 180, 145, 47	345, 186, 23, 22, 5	255, 225, 47, 41, 13
LMA1	Mmashoro 1	588	322, 166, 100	209, 185, 147, 47	345, 186, 29, 28	260, 225, 47, 41, 15
LMA3	Mmashoro 3	586	322, 160, 104	209, 185, 147, 47	345, 186, 33, 22	254, 225, 47, 41, 19
LMA4	Mmashoro 1	582	450, 102, 30	582	582	582
LMA5	Mmashoro 2	590	332, 100, 85, 73	396, 194	375, 215	590
LMB1	Mmashoro 1	584	322, 161, 101	209, 180, 148, 47	345, 186, 30, 23	255, 225, 47, 41, 16
MIAC1	Mmashoro 1	585	322, 162, 101	209, 181, 148, 47	345, 216, 24	256, 225, 57, 47
MIAC2	Mmashoro 1	585	322, 162, 79, 19, 3	209, 181, 99, 49, 47	345, 216, 24	225, 135, 121, 57, 47
MIAC3	Mmashoro 1	586	322, 160, 94, 6, 4	209, 179, 151, 47	345, 219, 22	254, 225, 60, 47
MIAC4	Mmashoro 2	587	322, 161, 104	209, 180, 151, 47	345, 219, 23	255, 225, 60, 47
MIIBC5	Mmashoro 2	582	322, 161, 104	209, 180, 99, 47, 47	345, 214, 23	280, 255, 47
P10	Palapye 1	583	322, 134, 124, 3	209, 184, 143, 47	333, 186, 64	225, 221, 90, 47
P11	Palapye 1	582	322, 134, 126	209, 184, 142, 47	332, 186, 64	225, 220, 90, 47
P13	Palapye 3	585	322, 161, 102	209, 180, 149, 47	345, 186, 31, 23	255, 225, 58, 47
P14	Palapye 2	581	322, 135, 124	209, 184, 140, 47	330, 186, 65	225, 218, 91, 47

P16	Palapye 2	580	322, 134, 124	209, 184, 140, 47	330, 186, 65	225, 218, 90, 47
P17	Palapye 3	586	322, 140, 125	209, 184, 140, 47	326, 186, 70, 5	225, 219, 96, 47
S2	Serowe 1	590	322, 169, 99	209, 188, 146, 47	345, 186, 31, 28	263, 225, 47, 41, 14
S6	Serowe 2	588	322, 165, 101	209, 184, 148, 47	345, 186, 30, 27	259, 225, 47, 41, 16
S7	Serowe 3	585	322, 163, 100	209, 182, 147, 47	345, 186, 29, 25	257, 225, 47, 41, 16
T5	Tonota 1	591	322, 165, 104	209, 184, 151, 47	345, 219, 27	259, 225, 47, 43, 17
T6	Tonota 1	594	322, 168, 104	209, 184, 151, 47	375, 186, 33	225, 135, 127, 47, 41, 19
T8	Tonota 2	584	322, 161, 101	209, 180, 148, 47	345, 186, 30, 23	255, 225, 47, 41, 15
T9	Tonota 2	583	322, 161, 100	209, 180, 147, 47	345, 186, 29, 23	255, 225, 47, 41, 15
T11	Tonota 3	592	322, 168, 102	209, 187, 149, 47	345, 186, 31, 30	262, 225, 47, 41, 17
Z2	Letlhakane 1	583	483, 100	209, 180, 147, 47	209, 186, 136, 29, 23	225, 135, 116, 56, 47, 4
WT	Strain from the fruits	580	322, 160, 104	209, 187, 137, 47	327, 187, 66	226, 215, 92, 47

Table C2. The non-*Saccharomyces* yeast strains PCR-RFLP of 26S region of the rRNA using three restriction endonucleases (*HaeIII*, *HinfI*, *GluI* and *RsaI*).

Isolate name	Location designation	Restriction patterns				
		AP ^a	<i>HaeIII</i>	<i>HinfI</i>	<i>GluI</i>	<i>RsaI</i>
AC4MIII	Mmashoro 1	590	322, 164, 81, 23	209, 183, 151, 47	345, 186, 33, 26	258, 225, 60, 47
L1	Letlhakane 1	572	237, 173, 159, 3	385, 100, 87	177, 169, 83, 59, 39, 19, 14, 12	298, 274
L2	Letlhakane 1	582	237, 176, 68, 65, 33, 3	392, 100, 90	169, 147, 67, 59, 39, 32, 23, 20, 14, 12	305, 277
L4	Letlhakane 1	575	237, 175, 68, 60, 33, 2	387, 100, 88	169, 147, 62, 59, 39, 32, 23, 19, 14, 11	300, 275
L9	Letlhakane 3	589	425, 164	210, 183, 147, 49	225, 188, 121, 29, 26	283, 259, 47
L12	Letlhakane 3	592	428, 164	210, 183, 150, 49	225, 188, 121, 32, 26	286, 259, 47
L13	Letlhakane 3	572	572	524, 48	396, 146, 30	516. 31, 25
LMB2	Letlhakane 3	576	177, 173, 162, 60, 4	388, 100, 88	183, 128, 79, 66, 49, 39, 20, 12	301, 275
LMB4	Letlhakane 1	574	177, 175, 162, 60	388, 100, 86	183, 128, 79, 66, 49, 39, 18, 12	301, 273
LMC1	Letlhakane 1	576	177, 176, 163, 60	389, 100, 87	183, 128, 79, 67, 49, 39, 19, 12	302, 274
LMC4	Letlhakane 1	572	177, 176, 159, 60	385, 100, 87	183, 128, 79, 63, 49, 39, 19, 12	298, 274
M2	Maun 1	569	237, 173, 91, 68	385, 101, 83	222, 128, 60, 59, 50, 23, 16, 11	298, 271
M3	Maun 1	568	237, 173, 90, 68	384, 101, 83	222, 128, 59, 59, 50, 23, 16, 11	297, 271

M4	Maun 2	579	419, 160	386, 146, 47	194, 188, 122, 28, 28, 26, 21	280, 252, 47
M7	Maun 3	597	410, 187	291, 193, 113	241, 214, 142	267, 204, 67, 46, 13
M8	Maun 3	581	418, 163	389, 145, 47	194, 185, 122, 28, 28, 24	264, 255, 47, 15
MIAC4	Mmashoro 3	587	322, 161, 104	209, 180, 151, 47	345, 219, 23	255, 225, 60, 47
P8	Palapye 2	605	586, 19	405, 200	325, 128, 93, 59	320, 271, 14
S1	Serowe 1	586	586	390, 147, 49	225, 188, 121, 29, 23	267, 256, 47, 16
S4	Serowe 1	587	587	210, 180, 148, 49	225, 188, 144, 30	284, 256, 47
S5	Serowe 2	573	369, 167, 22, 15	389, 102, 46, 36	450, 105, 18	573
S8	Serowe 8	572	572	524, 48	396, 146, 30	516, 31, 25
T1	Tonota 1	589	420, 169	256, 188, 89, 56	224, 186, 131, 27, 21	310, 225, 40, 14
T4	Tonota 1	595	423, 172	209, 191, 92, 56, 47	196, 186, 131, 30, 28, 24	266, 225, 47, 41, 16
T7	Tonota 1	591	427, 164	210, 183, 149, 49	225, 188, 121, 31, 26	285, 259, 47
T10	Tonota 2	571	177, 166, 159, 60, 9	385, 100, 86	183, 158, 79, 63, 49, 39	298, 273
T12	Tonota 3	587	587	390, 148, 49	225, 218, 144	303, 284
T13	Tonota 3	586	426, 160	210, 179, 148, 49	225, 188, 121, 30, 22	268, 255, 47, 16
T14	Tonota 3	568	177, 172, 159, 60	385, 100, 83	183, 128, 79, 63, 49, 39, 16, 11	298, 270
T15	Tonota 3	569	177, 173, 159, 60	385, 100, 84	183, 128, 79, 63, 49, 39, 17, 11	298, 271
T16	Tonota 3	584	423, 161	210, 180, 145, 49	225, 188, 121, 27, 23	267, 256, 47, 14
T17	Tonota 3	569	177, 173, 159, 60	385, 100, 84	183, 128, 79, 63,	298, 271

					49, 39, 17, 11	
T18	Tonota 3	567	177, 171, 159, 60	385, 100, 82	183, 128, 79, 63,	298, 269
					49, 39, 15, 11	
Z1	Letlhakane 1	595	409, 186	291, 192, 112	241, 213, 141	266, 203, 67, 46, 13

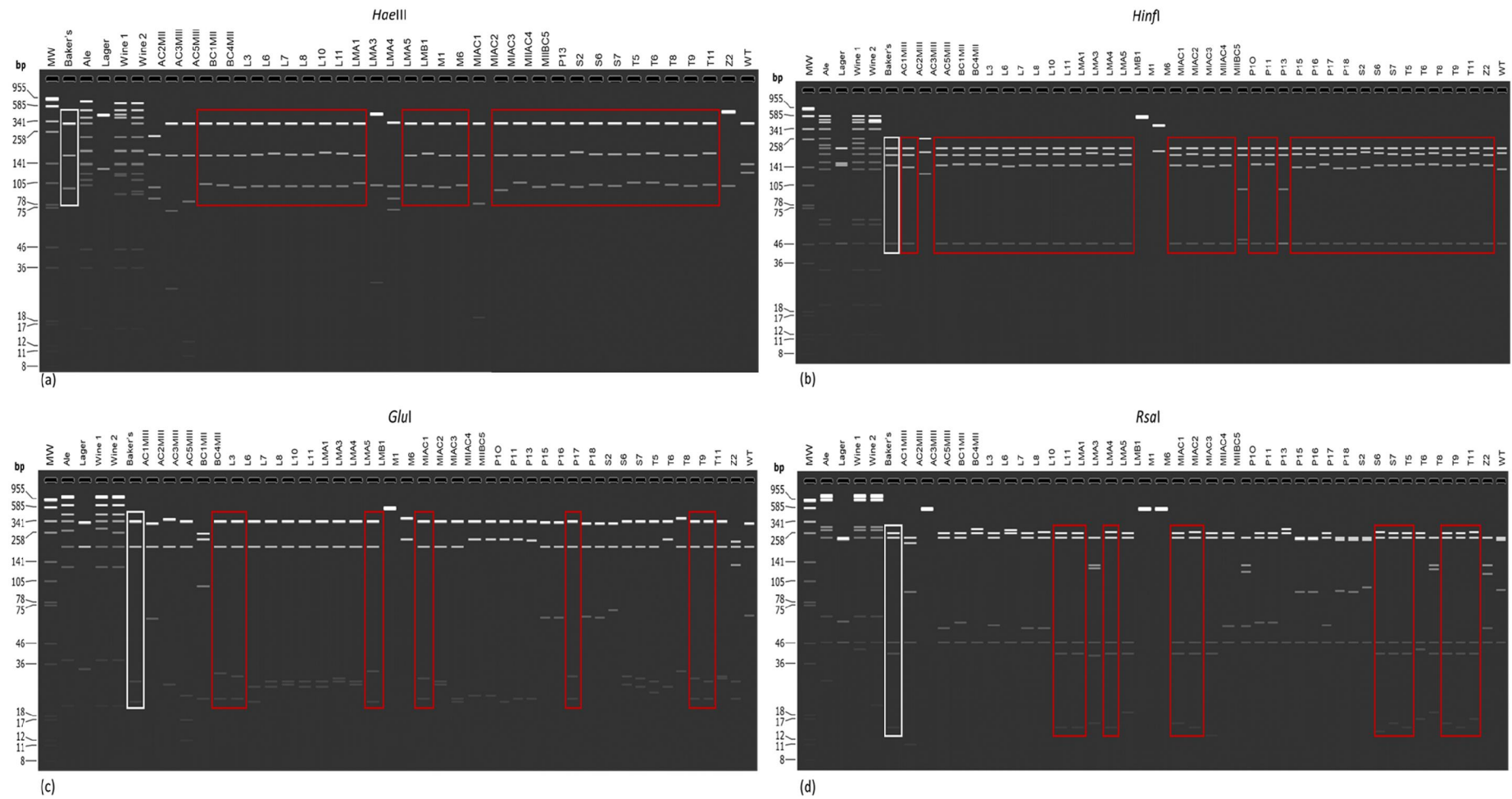


Figure C1: PCR-RFLP for the all *S. cerevisiae* isolates from *khadi* samples. The red shows yeast isolates similar to the control commercial baker's yeast (white boundary) while the WT is the wild type *S. cerevisiae* isolated from the *Grewia flava* fruits.

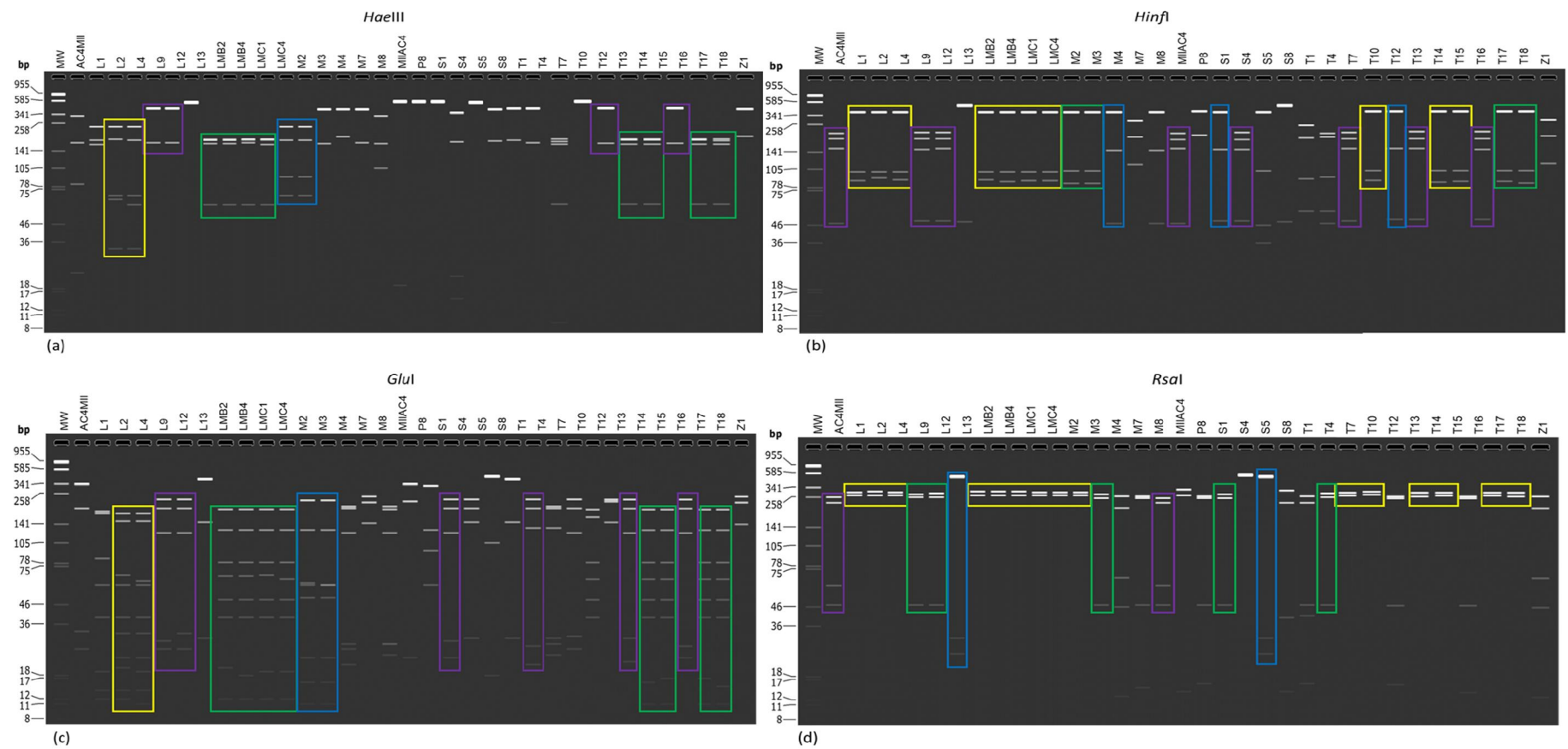


Figure B2: PCR-RFLP for the all non-*Saccharomyces* isolates from *khadi* samples. The restriction patterns of isolates indicate similarities among isolates from different regions of the country.

Appendix D

Table D1: The stress tolerance of yeast isolates from *Grewia flava* fruits and *khadi*.

Samples	Sugars (%)					Ethanol (%)				Salt (M)				pH				Temperature (°C)					
	YPS	YPS	YPL	YPF	YPM	3	5	7	9	0.5	1.0	1.5	2.0	1	2	3	5	37	40	41	42	43	
	(10)	(20)	(2)	(2)	(2)																		
D2	++	-	+	++	+	++	++	++	+	+	-	-	-	-	+	++	-	+++	+++	+++	+++	+++	
D3	++	++	+++	++	++	+++	++	++	+	+	+	-	-	-	+	++	+++	+++	+++	+++	+++	+++	
D4	-	-	+	+	-	+	++	++	+	+	+	-	-	-	+	++	-	+	+	+	+	+	
MA1	+++	+	-	++	+	+++	+++	++	+	+	-	-	-	-	++	++	+	+++	+	+	-	-	
MA2	++	-	++	-	++	-	-	-	-	-	-	-	-	-	-	+	++	+	+	-	-	-	
MA4	+++	++	+	++	++	++	++	+	+	+	+	-	-	-	+	+++	+++	++	++	++	+	+	
MA7	++	++	-	-	++	+++	++	++	++	+	-	-	-	-	+	+++	+	+++	+++	+++	+	-	
P8	+	+	+	+	+	++	+++	++	+	+	-	-	-	-	+	+++	+	++	+	+	+	+	
S1	+++	+++	++	+++	++	++	++	+	+	+	+	+	-	-	-	++	+	+	+	-	-	-	
S5	+++	++	++	+++	+++	++	++	+	+	+	-	-	-	-	-	++	+	-	-	-	-	-	
T1	++	+	-	++	-	++	-	-	-	+	-	-	-	-	-	-	-	++	+	+	-	-	
T17	+++	+++	++	+++	++	++	++	++	++	+	-	-	-	-	-	+++	-	-	-	-	-	-	
Z1	++	++	+	+++	++	+++	+	-	-	-	-	-	-	-	-	+	+	++	++	+	-	-	
Ctrl	+	++	++	+++	+++	++	++	++	++	+	+	-	-	-	-	+++	+++	+++	+++	+++	++	++	

Appendix E

Table E1: The ethanol assay test results for the *khadi* samples. The experiment was done in duplicates and repeated twice.

Sample	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Ethanol % (v/v)	Standard deviation
Letlhakane 1	6.664	6.236	6.486	6.759	6.536	0.230
Letlhakane 2	5.272	5.367	5.676	5.653	5.492	0.203
Letlhakane 3	5.772	5.831	5.902	5.855	5.840	0.054
Maun 1	8.639	8.425	8.818	8.628	8.628	0.161
Maun 2	4.236	3.987	3.558	3.891	3.918	0.281
Maun 3	0.762	0.940	0.738	0.678	0.779	0.113
Mmashoro 1	6.140	6.093	5.117	5.236	5.647	0.545
Mmashoro 2	7.354	7.497	6.652	6.724	7.057	0.431
Mmashoro 3	7.259	7.116	8.318	8.830	7.881	0.829
Palapye 1	8.068	8.068	8.366	8.235	8.184	0.144
Palapye 2	6.343	6.033	6.533	6.462	6.343	0.221
Palapye 3	7.783	7.973	6.807	6.973	7.384	0.580
Serowe 1	2.249	2.297	3.046	3.296	2.722	0.529
Serowe 2	1.416	1.416	1.380	1.595	1.452	0.097
Serowe 3	1.166	1.523	0.916	0.893	1.125	0.293
Tonota 1	4.403	4.320	4.915	4.831	4.617	0.299
Tonota 2	7.342	7.402	7.426	7.426	7.399	0.039
Tonota 3	4.284	3.903	3.820	3.879	3.972	0.211

Table E2: The pH of the *khadi* samples which was done in triplicates.

SAMPLE	TRIAL 1	TRIAL 2	TRIAL 3	Average	Standard Deviation
Letlhakane 1	3.540	3.540	3.540	3.540	0.000
Letlhakane 2	3.620	3.620	3.620	3.620	0.000
Letlhakane 3	3.320	3.320	3.320	3.320	0.000
Maun 1	3.020	3.020	3.030	3.023	0.006
Maun 2	2.740	2.740	2.730	2.737	0.006
Maun 3	2.970	2.970	2.970	2.970	0.000
Mmashoro 1	3.340	3.340	3.340	3.340	0.000
Mmashoro 2	3.310	3.310	3.310	3.310	0.000
Mmashoro 3	3.370	3.370	3.370	3.370	0.000
Palapye 1	2.710	2.710	2.700	2.707	0.006
Palapye 2	2.800	2.800	2.790	2.797	0.006
Palapye 3	2.640	2.640	2.650	2.643	0.006
Serowe 1	2.810	2.810	2.820	2.813	0.006
Serowe 2	3.610	3.610	3.610	3.610	0.000
Serowe 3	3.460	3.460	3.460	3.460	0.006
Tonota 1	2.820	2.820	2.830	2.823	0.006
Tonota 2	2.690	2.690	2.690	2.823	0.000