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OPTIMISATION OF FERMENTATION PROCESSES OF LOCAL CEREAL-BASED BEVERAGES TO PRODUCE IMPROVED CEREAL-BASED PROBIOTIC BEVERAGES

Malehe Cosmas Setta

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Life Sciences of the Nelson Mandela African Institution of Science and Technology

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ABSTRACT

Fermented cereal-based probiotic beverages are rare and have rarely been produced in Tanzania. Though the beverages are novel, the potentiality of such beverages has for some decades been exhibited through local fermented cereal-based beverages. In recent years there has been a paradigm shift towards non-diary probiotic products because of the negative health effects milk and dairy products have on the multitude of people around the world. A quantitative study employing purposeful sampling was carried out in three regions of Mbeya, Morogoro and Kilimanjaro whereby in each of the regions; quadruplicate samples of either of the traditional cereal-based beverages locally known as *Kindi, Kimpumu, Togwa* or *Mbege* were collected from a target village and stored at 4 °C in the laboratory. Identification of probiotic microbes in the local beverages was done and probiotic *Kindi, Kimpumu, Mbege* and *Togwa* were developed using pure cultures of the identified probiotic bacteria. Probiotic cultures used were *Lactobacillus brevis* for *Togwa*, *Lactobacillus plantarum* for *Kimpumu* and *Mbege* and *Pediococcus pentosaceus* for *Kindi*. After 24-48 h of controlled fermentation at 37 °C; results showed that the prepared cereal-based beverages were probiotic with mean viable cell counts of 1×10^{11} cfu/mL and mean pH 4.77. During storage the probiotic cereal beverages remained stable for five days at 25 °C and 28 days (4 weeks) at 4 °C with viable cell count of 2.0×10^{11} cfu/mL and pH 3.83 at 25 °C and viable cell count of 2.0×10^{11} cfu/mL and pH 4.08 at 4 °C, respectively. There was no growth of pathogens in the beverages. The four cereal-based probiotic beverages were equally accepted by consumers through a sensorial evaluation. This study shows that controlled fermentation of cereals using carefully selected probiotic bacteria results in probiotic cereal based-beverages with good quality attributes and safety and further advance the knowledge on fermented cereal substrates as nutrient-rich and promising delivery vehicles for probiotics by sustaining the growth of a large population of lactic acid bacteria.

DECLARATION

I, Malehe Cosmas Setta do hereby declare to the Senate of Nelson Mandela African institution of Science and Technology that this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

Malehe C. Setta

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The above declaration is confirmed

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Supervisor 1	**Signature**	**Date**

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CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by the Nelson Mandela African Institution of Science and Technology a dissertation entitled: *Optimization of Fermentation Processes of Local Cereal-based Beverages to Produce Improved Cereal-based Probiotic Beverages* in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Life Sciences of the Nelson Mandela African Institution of Science and Technology.

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DEDICATION

This work is dedicated to my wife Rachel and my children; Ivan, Noëlla, Ingrid and Nancy for their patience and perseverance.

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LIST OF ABBREVIATIONS AND SYMBOLS

AFB1	Aflatoxin B1
ANF	Antinutritional Factor
API	Analytical Profile Index
ARDRA	Amplified rDNA Restriction Analysis
As	Arsenic
BCCM-LMG	Belgian Coordinated Collection of Microorganisms-Laboratory of Microorganisms Ghent
Ca	Calcium
Cd	Cadmium
CFU	Colon Forming Units
CHOs	Carbohydrates
CO_2	Carbon Dioxide (gas)
SSA	Salmonella-shigella agar
BGA	Brilliant Green Agar
VRBGA	Violet Red Bile Glucose Agar
CREATES	Center for Research, Agricultural Advancement, Teaching Excellence and Sustainability in Food and Nutrition Security
CVD	Cardiovascular Disease
DNA	Deoxyribonucleic Acid
FAO	Food and Agriculture Organization
Fe	Iron
GIT	Gastrointestinal Tract
GRAS	Generally Regarded As Safe
H_2	Hydrogen Gas
Hg	Mercury
HIV/AIDS	Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome
JAR	Just-about-right (sensory test measure)
LAB	Lactic Acid Bacteria
MAC	MacConkey Agar
Mg	Magnesium
MLSA	Multilocus Sequence Analysis

Mo	Molybdenum
MRS	De Man, Rogosa and Sharpe Agar
N_2	Nitrogen Gas
NA	Nutrient Agar
NaCl	Sodium Chloride
Pb	Lead
PCR	Polymerase Chain Reaction
PFOF	Probiotic Fermented Oat Flour beverage
Ph	Measure of Hydrogen ion (H^+) Concentration
RFLPs	Restricted Fragment Length Polymorphism
RNA	Ribonucleic Acid
SDA	Sabourad Dextrose Agar
SUA	Sokoine University of Agriculture
UK	United Kingdom
UN IGME	The United Nations Inter-Agency Group for Child Mortality Estimation
USA	United States of America
USD	United States Dollar
WHE	Western Heads East
WHO	World Health Organization
Zn	Zinc

CHAPTER ONE

INTRODUCTION

1.1 Background of the problem

In African countries and other developing countries; cereals occupy a large share of the staple diets (40% or one-third) and these are mainly in form of fermented foods or beverages (Amadou, 2011; Kohajdová, 2016). Fermented cereal-based beverages are consumed regularly in social gatherings and other important community rituals (Aka *et al*., 2014; Kohajdová, 2016). Fermentation of cereal-based beverages involve beneficial microbes of diverse nature and originate from different genera of bacteria and fungi (Enujiugha & Badejo, 2017; Oyedeji *et al*., 2013). Lactic acid bacteria (LAB) has been reported to comprise a major genus of bacteria which are involved in cereal fermentation (Achi & Asamudo, 2019; Aka *et al*., 2014; Blandino *et al*., 2003; Mokoena *et al*., 2016; Mukisa *et al*., 2017) whereas the most predominant group of fungi involved in such fermentation is yeast (Mukisa *et al*., 2017; Ogunremi *et al*., 2015; Todorov & Holzapfel, 2014).

Spontaneous fermentation has been used in Tanzania and other African countries for preservation and processing of food for many centuries ago (Franz *et al*., 2014; Kohajdová, 2016). However, limited or little information is available on the type of microorganisms involved in such fermentation (Franz *et al*., 2014). Most of the fermented cereal-based beverages in Tanzania lack information on quality aspects and functionality when ingested in the human gut. Furthermore, and precisely, studies on microflora taking part in the fermentation of locally available cereal beverages in Tanzania are scarce and microbes isolated and identified from such beverages are also limited (Kalui *et al*., 2010; Mugula *et al*., 2003a). Spontaneous fermentation of cereal-based beverages in many parts of the world and particularly in Africa has invariably led to production of fermented cereal beverages with mixed cultures involving both beneficial (probiotics) and non-beneficial (pathogenic and spoilage) microflora (Achi & Asamudo, 2019; Aka *et al*., 2014; Enujiugha & Badejo, 2017). Besides, the fermented cereal products produced are of low quality, safety and microbial stability (Guyot, 2012). This is a major hurdle which makes most of the traditionally produced cereal-based beverages of lower market values compared to those produced under controlled fermentations using carefully selected microbial cultures.

Lactic acid bacteria which are mainly involved in the lactic acid fermentation of cereal-based foods and beverages have been found to impart health benefits when ingested together with the fermented cereal substrate and other end metabolites of the fermentation process (Achi & Asamudo, 2019; Enujiugha & Badejo, 2017; Kalui *et al*., 2010). A number of African traditionally fermented cereal-based foods and beverages such as Ogi, Pito, Togwa and Tchoukoutou have been found to be potentially probiotic because of the Lactobacillus *spp*. they contain (Aka *et al*., 2014; Kohajdová, 2016). Probiotic microbes are those which impart health benefits to the host when ingested besides the nutrients they provide by biologically acting on the substrates. Consumption of probiotics by human being has mainly been dependent on milk and milk products as delivery vehicles or media. Consumers health concerns on milk and milk products that include high cholesterol content, lactose intolerance, allergy, cultural taboos and religious beliefs have led to increased demand for non-diary probiotic products (Gupta & Bajaj, 2016; Nyanzi & Jooste, 2012). The health benefits derived from consumption of probiotics include prevention and cure of diabetes, cancer, CVD, obesity, allergy, acute diarrhoea and lactose intolerance (Min *et al*., 2019; Ouwehand & Röytiö, 2014; Ranadheera *et al*., 2017).

Despite the numerous health benefits probiotics have on human beings, milk and milk products for many decades have almost exclusively been used as delivery vehicles compared to other food substances (Bansal *et al*., 2015; Nyanzi & Jooste, 2012; Salmerón, 2017). However, milk and milk products have limitations such as lactose intolerance, cholesterolemia, allergy on some segments of the consumer market giving rise to research on alternative food carriers such as cereals (Nyanzi & Jooste, 2012). Research in functional foods like cereal-based probiotic beverages is driven by consumers desire for quality life, healthcare cost reduction and the preference for foods which are naturally preserving and health improving (Anukam & Reid, 2009; Kalui *et al*., 2010). Investigations have revealed that traditional African fermented cereal-based beverages are possible probiotic carriers because of the probiotic *Lactobacillus spp*. and yeasts which are involved in the fermentation of such products (Aka *et al*., 2014). As such, more focus is now on the traditional African fermented cereal beverages as sources of novel probiotics as well as avenues for development of new fermented cereal-based probiotic beverages to meet the growing demand for non-dairy versions (Kalui *et al*., 2010).

Development of fermented cereal based probiotic beverages from traditional African fermented cereal beverages provide accessible, low cost and acceptable probiotic products especially to rural people (Aka *et al*., 2014; Chaves-López *et al*., 2014; Franz *et al*., 2014). This study

attempted to develop cereal-based probiotic beverages by using pure cultures of probiotic microbes identical to those isolated from local cereal-based beverages known as *Kindi, Kimpumu, Togwa and Mbege*. The choice of these alternative delivery vehicles lies on the foundation that cereals and cereal products contribute more than 60% of the world's food production and provide the human being with essential nutrients such as proteins, carbohydrates, fiber, vitamins and minerals (Todorov & Holzapfel, 2014). Furthermore, the growth of the probiotic industry is expected to increase in the coming eight years because of the rise in global health awareness among people, efficacy of probiotics in addressing health concerns, expansion of functional food industry worldwide, advent of novel technologies in the field and growing disposable incomes among consumers (GrandViewResearch, 2018).

1.2 Statement of the problem

Milk and milk products have almost exclusively been used as delivery media for probiotic bacteria to human being for many decades (Nyanzi & Jooste, 2012; Salmerón, 2017). However, such dairy products have limitations that include cost (especially in the developing world), allergens, cultural food taboos, religious beliefs, lactose intolerance, high cholesterol content, vegetarianism, veganism, requirement of cold-chain facilities, and the need to use beverages that form part of the people's daily diets (Gupta & Bajaj, 2016; Nyanzi & Jooste, 2012). There is thus a need to research on alternative food matrices that can be used as delivery media for probiotics in order to meet demands and preferences of different consumers taking into consideration the increasing need to consume food for health reasons by avoiding those with negative health effects (Bansal *et al.*, 2015; Nyanzi & Jooste, 2012; Peres *et al.*, 2012). As such this study focused on researching the traditional fermented cereal-based beverages as sources of probiotics and possible carrier of probiotics to human being when the fermentation processes leading to their production are improved.

1.3 Rationale of the study

Local or commercial production of fermented cereal-based probiotic beverages and isolation, identification and screening of strains of probiotics are rare in Tanzania and many other countries particularly in sub-Saharan countries (Anukam & Reid, 2009; Di Stefano *et al.*, 2017). However, the main problem is lack of information if some of the local cereal-based beverages have probiotic potential. This study developed fermented cereal-based probiotic beverages that paved the way for production and consumption of the health-giving beverages

by local people and improve their well-being. The study developed such beverages because they are products which are similar to the local ones normally used by the people though they have added value by having probiotics. Many communities in Tanzania use spontaneously fermented cereal-based beverages for refreshment and this provides avenues for introduction of the cereal-based probiotic beverages among the society. The cereal-based probiotic beverages were developed as another milestone in the quest for alternative vehicles for probiotics delivery as well as pioneer the production and consumption of the products in the country. In Tanzania, cereal-based beverages with potential for development as probiotic products are traditional maize, millet or sorghum based alcoholic and non-alcoholic refreshments such as Togwa (Achi & Asamudo, 2019)

1.4 Research objectives

1.4.1 General objective

To develop cereal-based probiotic beverages (*Togwa, Mbege, Kindi, and Kimpumu*) through optimization of fermentation processes.

1.4.2 Specific objectives

(i) To isolate and identify microbes taking part in fermentations in the four samples of local cereal-based beverages.

(ii) To optimize the fermentation processes of the cereal-based beverages (*Togwa, Mbege, Kindi* and *Kimpumu*) using commercial Lactic Acid Bacteria identified as probiotic microbes under controlled conditions.

(iii) To assess the probiotic potential of the cereal-based beverages prepared in (ii) above.

(iv) To conduct sensory evaluation and shelf-life studies of the potential cereal-based probiotic beverages identified in (iii) above.

1.5 Research questions

(i) What kind of microbes participate in the fermentations of the four cereal-based beverages?

(ii) How can the local cereal-based beverages be formulated and optimized using pure starter cultures of the identified probiotics under controlled conditions?

(iii) Which of the cereal-based beverages prepared have probiotic potential?

(iv) Which of the potential probiotic beverages are acceptable and can retain their probiotic qualities?

1.6 Significance of the study

Consumption of fermented cereal-based probiotic beverages provide the majority of B-group vitamins to consumers, important micro-nutrients such as Fe, Zn, Ca, Mn, and Mg. The cereal-based probiotic beverages produced in this study have good organoleptic attributes such as appearance, color, taste, aroma, and texture and undergo self-bio preservation through production of organic acids and bacteriocins, which kills both spoilage and pathogenic bacteria. This makes the beverages produced using probiotics microbiologically safe to consume.

Fermented cereal-based probiotic beverages provide an alternative to people who cannot consume probiotics in a milk medium due to cultural taboos or religious beliefs, allergy, lactose intolerance, cholesterol content as well as those who prefer to consume such beneficial microbes in a medium of a staple food, they are accustomed to. Furthermore, development of fermented cereal-based probiotic beverages bridges the existing gap of more dependency on milk and milk products as delivery media of probiotics than any other food substance.

Furthermore, through use of locally available cereals such as maize and finger millet to prepare probiotic beverages; this study expands the market for such cereals and promote economic gains (income generation) to local cereal producers. Development of cereal-based probiotic beverages from local beverages implies value addition to staple foods customarily acceptable by people especially the rural majority. The market expansion of probiotic beverages also increases the incomes of cereal beverage producers. Furthermore, the study provides an alternative route for cereal use and cereal product diversification and consequently increase availability of cereal-based foods.

Production and consumption of cereal based-probiotic beverages is rare or non-existent in Tanzania. This study goes a long way in imparting knowledge and increase awareness on the benefits derived from consuming cereal-based probiotic beverages such as intake of B-group

vitamins and other micronutrients like Fe, Zn and Mn. The study also provides a potential substitute for milk-based probiotic foods as well as increased income generation for cereals and cereal-based beverage producers.

1.7 Delineation of the study

This study focused on isolation, characterization and identification of microbes taking part in the fermentation of the four cereal-based beverages, namely Kimpumu, Kindi, Mbege and Togwa collected in three respective regions in Tanzania; Mbeya, Kilimanjaro and Morogoro. Since only few locations were involved in this study, wide coverage of the study could have resulted to more species of microbes as environmental setting determines the kind of microorganisms available in the particular area.

Formulation of the fermented cereal-based probiotic beverages involved use of only three monocultures of LAB; *L. brevis, L. plantarum and P. pentosaceus*. Application of co-cultures with another probiotic LAB or yeast could possibly have produced more interesting results. Testing of the probiotic potential of the four formulated cereal-based beverages was only limited to 48 h of controlled fermentation at 37 °C; the length of controlled fermentations could further be prolonged to observe if any changes occur in the probiotic characteristics of the cereal-based beverages. The laboratory experimentation of the stability of the four-potential cereal-based probiotic beverages generated useful results. However, the possibility of experimentation for longer storage times/weeks would generate more useful information that could help improve keeping quality of the products.

CHAPTER TWO

LITERATURE REVIEW

2.1 Probiotics

Probiotics have been used by human beings from time immemorial even before the discovery of microbes. Ancient Egyptians and Tibetan nomads used fermented milk products and fermented yak milk respectively (McFarland, 2015). However, in the 1800s scientists began noticing the health effects of consuming fermented milk but no reason was given. During the same period a renowned scientist Louis Pasteur discovered bacteria and yeasts inducing fermentation in milk but he was unable to describe the health effects they impart in the human body (McFarland, 2015). It was only Elie Metchnikoff who in the 1860s was able to link the number of years the Bulgarians live to the presence of *Lactobacilli* in the gut and the fermented milk they consume (McFarland, 2015; Sánchez *et al*., 2017). Henry Tissier; in 1906 was able to isolate *Bifidobacterium* from an infant (McFarland, 2015).

Human studies in 1922 and 1932 involving the use of *Lactobacillus acidophilus* established the positive health effects in patients with chronic constipation, diarrhea, and mental disease. In the same time period (1930s); yoghurt starter culture (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) was abandoned in favor of *L. acidophilus* due to inability to colonize the human intestines (McFarland, 2015). In the 1940s; microbiological research focused on identification of pathogenic bacteria rather than probiotic strains of bacteria or yeasts. The focus of research on beneficial microbes in the following years (1950s- 1980s) centered on screening potential probiotic strains isolated from nature and human being as well. The modus operandi for probiotic strains was also defined (McFarland, 2015). Further research on probiotic strains shed more knowledge on the complex interactions of colon flora and their bactericidal actions against colonization by pathogenic bacteria (McFarland, 2015; Sánchez *et al*., 2017). In a nut shell, more effective research on probiotics was started in the decade 1980-1989 through clinical trials, and by 1990-1999 resulted to a few hundred publications (McFarland, 2015). The study of probiotics gained momentum from the period 2000 to 2014 during which there was an increase in the number of publications (McFarland, 2015).

Probiotics is a term coined by Lilley and Stillwell in 1965 to refer to substances secreted by one microorganism which in turn invigorates the growth of another (McFarland, 2015; Iqbal *et*

al., 2014; Sánchez *et al*., 2017; Kumar *et al*., 2015). The term was derived from Latin and Greek; simply meaning "for life" (Kandylis *et al*., 2016; Iqbal *et al*., 2014).

The Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) joint Working Group defined probiotics as "live micro-organisms, which when administered in adequate amounts confer a health benefit on the host"(FAO/WHO, 2002; Gil-Rodríguez *et al*., 2015; Marsh *et al*., 2014; WHO, 2001). *Lactobacillus* and *Bifidobacterium* are the two genera of Lactic acid bacteria (LAB) to which most of the known probiotic species belong (Blandino *et al*., 2003; Charalampopoulos *et al*., 2002; Gil-Rodríguez *et al*., 2015; Kandylis *et al*., 2016; Nyanzi and Jooste, 2012; Tur & Bibiloni, 2016). Other genera to which probiotic LAB belong are *Lactococcus, Leuconostoc, Enterococcus* and *Streptococcus* (Mokoena *et al*., 2016). Probiotic microorganisms should be adequately and regularly ingested to a threshold value of 10^6 cfu/mL per 100 mL serving for maintenance of intestinal microbiota as well as impart the health benefits to the consumer (Angmo *et al*., 2016; Mokoena *et al*., 2016; Nyanzi & Jooste, 2012; Peres *et al*., 2012; Shori, 2016). The benefits of ingestion of probiotics include prevention and treatment of infantile diarrhoea, travelers' diarrhoea, antibiotic induced diarrhoea, colon cancer, constipation, hypercholesterolemia, lactose intolerance, vaginitis and intestinal infections (Angmo *et al*., 2016; Nyanzi and Jooste, 2012). In recent years, the World Gastroenterology Organization has reaffirmed that the efficacy of probiotics is strain-specific and dose-specific (McFarland, 2015; Sánchez *et al*., 2017).

2.2 Types of probiotics

In 2014, three broad categories of probiotics were defined (McFarland, 2015). However, selection of probiotic product largely relies on the type of bacteria and kind of anticipated beneficial health effect (Iqbal *et al*., 2014). Table 1 summarizes the broad categories of probiotics.

Table 1: Categories of probiotics

SN	Type of probiotics	Role	Reference
1.	Generally regarded as safe (GRAS)	Not associated with any health claims; e.g., *Lactobacillus bulgaricus* and *Streptococcus thermophilus* used in fermentation of milk	McFarland (2015) Di Stefano *et al*. (2017)
2.	Food supplements	Associated with health claims; e.g., *Lactobacillus rhamnosus* GG used to improve normal colonic flora	McFarland (2015)
3.	Drugs	Curing of diseases; e.g., *Lactobacillus acidophilus* capsules used to cure acute pediatric diarrhea	McFarland (2015)

Potential probiotic strains must be screened to get the ones which can survive the GIT environment. Normally, probiotics candidates should survive as they move to the target organ, able to interact with the immune system, able to kill pathogenic bacteria, safe for human consumption, and industrially advantageous (Enujiugha & Badejo, 2017; Gil-Rodríguez *et al*., 2015; McFarland, 2015). Animal models and human volunteers are used in evaluating the kinetics, oral dose recovery, adherence to mucosal layer and persistence in the complex GIT environment (Mokoena *et al*., 2016). When identification of potential probiotic strain is done, *in vitro* or *in vivo* studies may follow to test resistance to gastric and bile acidity (Derrien & Van-Hylckama, 2015; Mokoena *et al*., 2016).

A good probiotic strain with high functionality must be acid tolerant, bile tolerant, oxygen tolerant, heat tolerant, ability to grow in food substrate and being able to metabolize prebiotics effectively as well as being appropriate for large-scale industrial production (survive food processing and storage conditions) (Gupta & Bajaj, 2016). In practice, microencapsulation, oxygen-impermeable containers, incorporation of nutrients and use of stress-resistant strains are employed to enhance the quality and functionality of probiotic strains (Mishra & Mishra, 2012). Consequently, a number of probiotic strains have found their way into the market through extensive screening and testing for their efficacy.

2.3 Probiotics products available in Africa

Information available on the market share of probiotics is very limited. In global terms the market share in Africa is minimal (Franz *et al*., 2014; GrandViewResearch, 2018; Mukisa, 2016). Some sort of a well-established market can be seen in South Africa (Franz *et al*., 2014).

In Tanzania; literature does not show noticeable consumption of probiotics in any delivery media. The only effort to use probiotics in Tanzania was done by the Western Heads East (WHE) project at Mabatini, Mwanza region in 2004 through use of probiotic yoghurt to treat victims of HIV/AIDS (Reid, 2010). Previously, an attempt was done by Willumsen *et al*. (1997) of managing acute diarrhea in children by use of fermented and amylase-digested weaning gruel. This indicates that knowledge and awareness of the existence of probiotics and presence of edible probiotic products in most of the African countries is very minimal.

2.4 Lactic Acid Bacteria

Lactic acid bacteria belong to the groups of bacteria in the phylum Firmicutes, class I of Bacilli, order II of Lactobacillales, family I of Lactobacillaceae and genus I of *Lactobacillus* (Hammes & Hertel, 2009). The family Lactobacillaceae consists of three genera namely *Lactobacillus, Paralactobacillus and Pediococcus*. This is a group of microorganisms (bacteria) which are generally gram-positive, non-motile, non-spore forming, non-respiring, catalase-negative cocci or rods and during carbohydrate fermentation they yield lactic acid as the main end product (Hammes & Hertel, 2009; Waters *et al*., 2015). They can survive at very low acid condition (pH 5 and lower). The major groups or genera which are regarded as lactic acid bacteria are *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Carnobacterium, Enterococcus, Oenococcus, Aerococcus, Vagococcus, Tetragenococcus* and *Weissella* (Waters *et al*., 2015). The *Lactobacilli* grow well under anaerobic conditions or reduced oxygen tension provided that there are sufficient carbohydrates, peptides, amino acids, nucleotides and vitamins (Hammes & Hertel, 2009).

The genus *Lactobacillus* is characterized by cell morphology; long, straight or slightly crescent rods and others being coryneform coccobacilli. Certain gas producing *Lactobacillus spp.* exist as a blend of long and short rods. *Lactobacilli* have a cell wall structure made up of peptidoglycan (murein) of different chemotypes of the cross-linkage group A. All *Lactobacilli* are gram-stain positive (Hammes & Hertel, 2009). When plated on agar media; colonies are normally small (≤ 5mm) having entire margins, smooth, convex, sparkling and opaque (Paul De Vos *et al*., 2009). However, growth in a liquid medium occurs entirely in the liquid though the microbial cells sediment after end of growth. The *Lactobacillus* cells don't develop any unique smell when cultivated in common media. Besides, these microbes when proliferate in food; their carbohydrate metabolism produce volatile compounds such as diacetyl, acetic acid and acetaldehyde while their amino acid (protein) catabolism leads to such compounds as

amines, hydrogen sulphide (H_2S), carbonyl compounds, benzaldehydes, methane-thiol and cresol (Hammes & Hertel, 2009).

Nutritionwise, *Lactobacilli* need a variety of nutrients for their growth. They are extremely fastidious microorganisms which require carbohydrates for energy, carbon sources, nucleotides, vitamins and amino acids. Usually, when media contain fermentable carbohydrates, meat and yeast extracts, and peptone; the necessary nutrient requirements are met. The most commonly used medium for cultivation of *Lactobacilli*; De Man, Rogosa and Sharpe Agar (MRS agar) contains all the various requirements of the essential nutrients needed by the microbes (Hammes & Hertel, 2009). The microbes initially grow best at a pH range of 6.4 to 5.4 and growth ends in the pH range 3.6-4.0 though this is species and strain specific. Examples of *Lactobacillus spp*. which exhibit acid (pH) tolerance are *Lactobacillus plantarum* and *Lactobacillus casei.* The majority of *Lactobacilli* grow well in a mesophilic temperature range of 25 – 40 °C. However, some may grow below 15 ºC and certain strains below 5 °C.

Protocol for enrichment and isolation of *Lactobacilli* has to take on board aciduric nature of the microbes, their complex nutritional requirements as well as microaerophilic growth conditions (Hammes & Hertel, 2009). When confirmed that the source sample is dominated by *Lactobacillus spp*; the non-selective De man, Rogosa and Sharpe agar (MRS agar) is used for the isolation of *Lactobacilli*. The MRS agar was developed primarily for the cultivation of *Lactobacilli* though some other LAB such as *Pediococcus* may also grow (Hammes & Hertel, 2009). Purification of the microbes is thus essential. Yeasts and molds as accompanying microflora may be removed by addition of cycloheximide (Hardy Diagnostics, 2019; Kurtzman *et al*., 2011). Generally, the addition of various concentrations of selective agents, such as cycloheximide, polymyxin, thallium acetate, sorbic acid, acetic acid, or sodium nitrite to the medium inhibit the growth of most of the accompanying microorganisms (Hardy Diagnostics, 2019). When cultivated; *Lactobacilli* grow better in anaerobic or increased CO_2 tension environment. As such agar plates are incubated in jars that have been evacuated and filled with 90% N_2 or H_2 plus 10% CO_2 or in anaerobic jars (BBL, Oxoid) employing CO_2 and H_2 generating kits (Hammes & Hertel, 2009). Isolation, characterization and identification of *Lactobacilli* can be achieved by employing standard methods applied by various researchers such as Abegaz (2007), Bennani *et al*. (2017), Deshpande *et al*. (2017), Fguiri *et al*. (2015), Kavitha *et al*. (2016) and Cho *et al*. (2018), just to mention a few.

2.4.1 Genus *Lactobacillus* groupings

Studies based on the 16S rRNA sequence analysis has established that the genus *Lactobacillus* can be subdivided into seven phylogenetic groups namely; *L. casei* group, *L. plantarum* group, *L. salivarius* group, *L. delbrueckii* group, *L. sakei* group, *L. buchneri* group, and *L. reuteri* group (Hammes & Hertel, 2009).

A new method for grouping *Lactobacillus spp*. has been developed based on the fermentation types. *Lactobacillus spp*. are allotted into three groups; A, B and C (Hammes & Hertel, 2009). Organisms placed in group A are obligate homofermentative *Lactobacilli* that ferment hexoses completely to lactic acid but not pentoses and gluconates. Group B consists of *Lactobacilli* that are facultative heterofermenters and ferment hexoses entirely to lactic acid and some species to lactic acid, acetic acid, ethanol and formic acid. Fermentation of pentoses to lactic acid and acetic acid is done with an inducible phosphoketolase (Hammes & Hertel, 2009). Group C is made up of obligate heterofermentative *Lactobacilli* which use the phosphogluconate pathway to ferment hexoses to lactic acid, acetic acid or ethanol, and CO_2. However, they use the pentose phosphate pathway to ferment pentoses to lactic acid and acetic acid (Hammes & Hertel, 2009).

2.4.2 Fermentation of cereals by lactic acid bacteria

Cereals are considered as ideal fermentable substrates for proliferation of probiotic LAB (Lamsal & Faubion, 2009; Waters *et al*., 2015). Non-digestible carbohydrates are present in cereals and these selectively stimulate the growth of probiotic bacteria (Marco *et al*., 2017; Mudgil & Barak, 2013). Fermentation of cereals by LAB leads to synthesis of flavor compounds as well as protein hydrolysis; both of them having an effect on texture and flavor of product (Marsh *et al*., 2014; Mugula *et al*., 2003c; Peyer *et al*., 2016). Fermentation of cereal beverages by LAB leads to beneficial production of vitamins, antimicrobials, organic acids, prebiotics, amino acids, volatile flavor compounds, as well as detoxifying and anti-allergenic effects to the end product (Marco *et al*., 2017; Nyamete, 2016; Ranadheera *et al*., 2017; Rezac *et al*., 2018; Waters *et al*., 2015). Traditional spontaneously fermented cereal-based beverages have improved shelf-lives, enhanced taste and nutrition (Achi & Asamudo, 2019; Kumari *et al*., 2015).

2.4.3 Genotypic methods for identification of lactobacilli

In recent years, genotypic identification of *lactobacilli* has gained more popularity as it is considered to give most reliable results (Hammes & Hertel, 2009). Most frequently, comparative analysis of 16S/23S rRNA gene sequences of *lactobacilli* are employed for immediate identification of unknown isolates. However, some *Lactobacillus spp*. depict high similarity of the 16S rRNA sequences. In such circumstances, multilocus sequence analysis (MLSA) of conserved protein-coding loci may come to a rescue (Hammes & Hertel, 2009). Besides the shortcoming narrated above, analysis of 16S rRNA sequences remains the rapid and dependable tool for identification of an unknown *Lactobacillus* isolate (Segole'ne *et al*., 2002). The DNA spacer sequences between the rRNA genes of *lactobacilli* vary greatly though adequately conserved for species-specific PCR primers construction. Comparison of 16S-23S rRNA gene intergenic spacer region sequences is also useful for species relatedness studies (Hammes & Hertel, 2009). Another method for species identification is the amplified rDNA restriction analysis (ARDRA) which relies on the restriction length polymorphism of 16S rRNA gene fragments amplified by PCR (Hammes & Hertel, 2009).

2.5 Yeasts/Fungi

Yeasts are ubiquitous and exist in various materials such as soil, water, plants and plant products, animals, skin, gastro-intestinal tract of animals and fermented foods (Lara-Hidalgo *et al*., 2017). They are simply viewed as unicellular fungi and differ from molds which are multicellular fungi. Yeasts differ from bacteria by having oval, elongated, elliptical or spherical cell shapes which are larger in size ranging from 5-8 µm in diameter (James *et al*., 2005). Yeasts grow in acidic conditions; over a wide range of acid pH as well as in ethanol (up to 18%). Many of them grow when there is 55 - 60% sucrose. They produce a variety of colors such as creamy, pink and red (James *et al*., 2005). Most yeasts found in foods divide by fission or budding. The fungal kingdom to which yeasts belong; is the second largest group of eukaryotic organisms on earth and their number is estimated at 1.5-5.1 million species (Raja *et al*., 2017; Schoch *et al*., 2012).

Yeasts are economically important to human life. Their major role in the production of fermented foods and alcoholic beverages (e.g., bread, beer, wine) can't be overlooked (Hierro *et al*., 2004; Raja *et al*., 2017). They also have ability to produce beneficial secondary metabolites like vitamins and antibiotics (Hierro *et al*., 2004; Raja *et al*., 2017). On the other

hand, yeasts are harmful as they can cause spoilage which results to huge economic losses in the food chain (Hierro *et al*., 2004). That is why in fermented products they are enumerated to determine shelf life.

Traditionally, yeasts have been classified based on their morphological, physiological and biochemical traits (de Barros Lopes *et al*., 2009; Hierro *et al*., 2004; Kurtzman, 2011) but new development in molecular biology has resulted to a number of DNA-based tools for characterizing and identifying yeasts (de Barros Lopes *et al*., 2009; Hierro *et al*., 2004; Raja *et al*., 2017). Such techniques embrace DNA-DNA hybridization, RFLPs of chromosomal DNA, ribosomal DNA sequencing, sequencing of the internal transcribed spacer (ITS) region, chromosome length polymorphism (CLP) and pulsed field gel electrophoresis (Johansen *et al*., 2019; Kurtzman *et al*., 2011). With these PCR-based methods, it is possible to differentiate species among themselves and identify species of yeast isolates (Hierro *et al*., 2004; Kurtzman, 2011).

Yeasts in spontaneously fermented cereal-based beverages, may originate from the raw materials (cereals, water), beverage handlers, processing equipment and surrounding environment (Johansen *et al*., 2019; Lara-Hidalgo *et al*., 2017). The functional properties of yeasts in African fermented food and beverages comprise stimulation of LAB, carbohydrate fermentation, production of flavor compounds, production of tissue-degrading enzymes, removal of mycotoxins and imparting probiotic health benefits (Johansen *et al*., 2019). In a study by Johansen *et al*. (2019), *S. cerevisiae* was found to be the major yeast species isolated from 43 indigenous sub-Saharan African fermented food and beverages, *Pichia kudriavzevii* being the second and *K. marxianus* the third. It has been reported that *S. cerevisiae* occurs most frequently in all indigenous African fermented food and beverages and where it occurs dominate all other yeast species. It dominates fermentation in 93% of all alcoholic beverages in Africa (Johansen *et al*., 2019). Similarly, the fermentation of non-alcoholic beverages is dominated by *P. kudriavzevii* whereas *C. tropicalis and K. marxianus* are invariably found in African fermented food and beverages. Yeasts also take part in the production of pozol; a fermented cereal-based beverage in South-eastern Mexico, made from maize dough known as nixtamal. The species involved include *Rhodotorula minuta, Debaryomyces hansenii, Candida guilliermondii* and *Kluyveromyces lactis*.

During fermentation of African cereal-based food and beverages, a lot of microbial interactions take place in the medium. The interactions can be competition, antagonism, mutualism,

commensalism and parasitism (Johansen *et al*., 2019; Lara-Hidalgo *et al*., 2017). There is normally co-existence of yeasts and LAB during spontaneous fermentation. However, this interaction is for mutual benefit whereby lactic acid produced by fermenting LAB favours the growth of yeasts and growth of LAB is supported by the amino acids, peptides and vitamins (B_6) produced by yeasts (Aka *et al*., 2014; Johansen *et al*., 2019; Lara-Hidalgo *et al*., 2017). This is the importance of yeasts in spontaneous cereal fermentation. Co-existence of yeasts and LAB is a characteristic of the spontaneously fermented non/low alcoholic cereal-based beverages. Similarly, several interactions between several groups of microorganisms occur, i.e., yeast-yeast, bacteria-bacteria, yeast-bacteria and yeast-mold (Johansen *et al*., 2019).

Yeasts presence in cereal-based beverages produce phytase enzyme that is important in the GIT for break-down and release of phosphorus and other divalent ions such as Fe^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+} and Mg^{2+} (Lara-Hidalgo *et al*., 2017; Moslehi-Jenabian *et al*., 2010). They have shown resistance to gastric conditions by surviving to pH as low as 1.5 - 2.5 and showing good viability in the presence of bile salts; properties characteristic of probiotics (Johansen *et al*., 2019; Lara-Hidalgo *et al*., 2017). Yeast *spp*. with probiotic characteristics such as *S. cerevisiae* and *S. cerevisiae* ssp. *boulardii* have been used in fermented cereal-based beverages to provide beneficial health effects (Lara-Hidalgo *et al*., 2017; Moslehi-Jenabian, Pedersen, & Jespersen, 2010). *P. Kudriavzevii* has shown probiotic potential in cereal beverages and has been found to inhibit cholesterol absorption in the human and thus prevent risks of coronary heart disease (Lara-Hidalgo *et al*., 2017).

Most of the African spontaneously fermented food and beverages are recognised for their natural flavors and tastes. In these beverages; yeasts are recognised for their role of development of flavor compounds by conversion of carbohydrates (Johansen *et al*., 2019; Mukisa *et al*., 2017). The major flavor compounds released by yeasts during fermentation of cereal beverages comprise organic acids, aldehydes, alcohols, and esters (Johansen *et al*., 2019; Lara-Hidalgo *et al*., 2017; Mukisa *et al*., 2017). For instance, the flavour compounds identified in cereal-based Togwa and Obushera include acetaldehyde, ethanol, acetoin, diacetyl, 2-methylbutanal, 3-methylbutanal, 2-methylpropanal, 2-methylpropan-1-ol, 2-methylbutan-1-ol, and 3-methylbutan-1-ol (Johansen *et al*., 2019; Mugula *et al*., 2003a).

Production of vitamin B_9 (folate) by yeasts during fermentation of cereal-based beverages is one way of improving the nutritional value of cereal-based products (Johansen *et al*., 2019; Lara-Hidalgo *et al*., 2017). Contrary to yeasts, the human body can't synthesize vitamin B_9 and

relies on intake from daily diet. Some of the Yeast *spp*. that produce folate include *S. cerevisiae, P. kudriavzevii, K. marxianus,* and *Weisella anomalus* (Greppi *et al*., 2017; Johansen *et al*., 2019). Folate is important in the body particularly for infants in preventing megaloblastic anaemia and neural tube defects. Suboptimal intake of folate may lead to cardiovascular diseases (CVD) and some cancers (Aka *et al*., 2014; Angelov *et al*., 2018; Hjortmo *et al*., 2008). In synergistic action with LAB, some yeasts have been found to carry out biological decontamination by reducing the hazardous effects of mycotoxins in African fermented cereal foods and beverages. Strains of *S. cerevisiae* have been reported to reduce mycotoxins in African fermented cereal beverages (Johansen *et al*., 2019) and there is possibility for antagonistic yeasts to be used to inhibit the growth of pathogenic bacteria (Lara-Hidalgo *et al*., 2017).

Some Yeast *spp*. are opportunistic pathogens and thus caution should be taken when selecting yeast strains for use as starter cultures. Generally, most *Candida spp*. are innocuous but some of them may proliferate in the host and induce diseases (Candidiasis) particularly in individuals with weakened immune system (immunocompromised) (Johansen *et al*., 2019). To date, almost 41 - 47% of total yeast infections globally is caused by *C. albicans* (Johansen *et al*., 2019).

2.6 Importance of food fermentations

2.6.1 Fermentation as a food processing and preservation method

Fermentation of cereals has been used as a food processing and preservation technology in many parts of the world since the dawn of human civilization; thousands of centuries ago (Guyot, 2012; Mokoena *et al*., 2016). As a food preservation technology; fermentation not only extends the shelf life of foods but also improves palatability, nutritive value, organoleptic properties, safety, digestibility and acceptability of traditional foods (Kumari *et al*., 2015; Mokoena *et al*., 2016; Mugula *et al*., 2003c; Shori, 2016). It is simple, inexpensive and of economic importance particularly for Africa. One of the oldest known fermentation processes is the lactic acid fermentation (Kohajdová, 2016; Nyamete, 2016). This is the most important and economical method of food production and preservation for human consumption (Desiye & Abegaz, 2013; Kohajdová & Karovičová, 2007). LAB are the main agents in the lactic acid fermentation of African foods and beverages and are popularly referred to as "generally regarded as safe (GRAS)" (Kalui *et al*., 2010; Mokoena *et al*., 2016; Mugula *et al*., 2003c). Lactic acid fermentation comprises of the chemical changes in foods accelerated by enzymes

of LAB resulting in a variety of fermented foods (Halami, 2017). An updated list (by 2012) of microbial species utilized in food fermentations is given by Bourdichon *et al.* (2012). Fermented foods constitute one-third of food intake by humans (Amadou, 2011; Kohajdová, 2016).

It is reported that African cereal-based fermented foods and beverages such as fermented Uji or gruel (Nyanzi & Jooste, 2012) and Togwa (Enujiugha & Badejo, 2017; Kohajdová & Karovičová, 2007; Mugula *et al*., 2003b) have a probiotic potential because of the probiotic *Lactobacillus spp*. contained in them, with some originating from human intestines. Beneficial cultures can enhance the quality of certain African fermented foods. It is reported that a fermented cereal gruel in Tanzania reduced diarrhea by 40% in consuming children compared to those children that did not consume it over a period of 9 months (Anukam & Reid, 2009; Marshall & Mejia, 2012; Nyanzi & Jooste, 2012). This was attributed to better beverage microbial safety as well as protection against intestinal enteropathogenic colonization (Willumsen *et al*., 1997). In one previous review; it was revealed that fermented cereal-based products which contained *Lactobacillus spp.* and lactic acid have viricidal, anti-leukemic, antitumor and antibacterial activities (Kalui *et al*., 2010; Nyanzi & Jooste, 2012; Olasupo *et al*., 2010).

Organic acids such as lactic, acetic, propionic and butyric acids are released as byproducts during lactic acid fermentation to obtain fermented cereal beverages and these lower the pH to levels of 3 to 4 and improve the flavour of the product (Kohajdová & Karovičová, 2007). The growth of a wide range of pathogens such as *Shigella*, *Salmonella* and *E. coli* is inhibited by the presence of undissociated forms of the acetic and lactic acids at low pH (Blandino *et al*., 2003; Nyanzi & Jooste, 2012) and thus improve food safety and extend the food shelf life.

Fermented foods, unlike non-fermented foods, have a longer shelf-life, making fermentation a key factor in the preservation of such foods (Todorov & Holzapfel, 2014). Generally, shelf-life, nutritional value, texture, taste and aroma of food products can be improved by fermentation (Aloys & Angeline, 2009; Kumari *et al*., 2015). Fermentation is thus, a principal food processing and preservation technology.

2.6.2 Fermentation as a means of removing antinutritional factors

Fermentation of cereals by lactic acid bacteria (LAB) brings about decrease of antinutritional factors (Chaves-López *et al*., 2014). Anti-nutritional factors (ANFs) include phytic acid,

tannins, oxalates, and phenolic acids which are polyphenols and are found in cereals and legumes and the foods prepared from them (Aka *et al*., 2014). Poor protein digestibility and limited mineral bioavailability promoted by ANFs may lead to malnutrition and stunted growth (Aka *et al*., 2014; Olasupo *et al*., 2010). Chelation of phosphorus and other minerals such as Ca, Mg, Fe, Zn, and Mo by phytic acid is a common phenomenon in cereals and legumes (Kohajdová & Karovičová, 2007; Waters *et al.,* 2015). Removal or reduction to lower levels of ANFs in some cereals can be achieved through fermentation by certain LAB and yeasts (Olasupo *et al*., 2010; Soro-Yao *et al*., 2014; Waters *et al*., 2015). It has been reported that strains of *L. plantarum* were able to degrade phytic acid in the cereals (Llamas-Arriba *et al*., 2019). Consequently, bioavailability of minerals such as Fe, Zn and Ca were enhanced. A reduction of phytate content by 39% in cereals has been observed through fermentation alone. A decrease of 88% of the phytates in cereal beverage was observed through the synergistic effect of fermentation and exogenous phytase (Llamas-Arriba *et al*., 2019; Nyanzi and Jooste, 2012). This shows that ANF in cereal foods can be minimized through careful fermentation adopting appropriate probiotic culture. Antinutrient compounds in cereals such as tannins and trypsin inhibitors render protein indigestible but fermentation makes the protein digestible (Kumari *et al*., 2015; Waters *et al*., 2015). Provision of fermented beverages to infants/children in Africa and other developing countries can mitigate the problem of malnutrition through improved levels of nutrients in the fermented products (Llamas-Arriba *et al*., 2019; Waters *et al*., 2015).

2.6.3 Fermentation and reduction of toxins in cereal products

Empirical results have shown that toxic compounds in cereal products are eliminated by microbial activity during fermentation process (Nyanzi & Jooste, 2012; Waters *et al*., 2015). Lactic acid fermentation is one of the novel strategy for reducing or removing mycotoxins in cereal foods (Ahlberg *et al*., 2015; Russo *et al*., 2017). It is also capable of inducing preservative and detoxifying effects on cereal foods (Chelule *et al*., 2010; Nyamete, 2016). Certain strains of LAB are able to subdue mycotoxins and, in the process, produce simpler metabolites (low molecular weight) or bind the toxins to bacteria cell walls (Ahlberg *et al*., 2015; Nyamete, 2016; Soro-Yao *et al*., 2014). Consequently, fermentation is anticipated to be one of the strategies employed to mitigate health risks related with exposure to mycotoxins in cereals and their products (Ahlberg *et al*., 2015; Aiko *et al*., 2016; Nyamete, 2016).

It has also been reported that food beverage preparation, which involves cooking after fermentation, together with the highly acidic conditions of the fermented food beverage, may physically alter the microbial cell structure thereby increasing the binding sites for mycotoxins such as Aflatoxin B_1 (AFB_1) (Ahlberg *et al*., 2015; Nyanzi & Jooste, 2012). This provides a way of reducing aflatoxins in African fermented foods and beverages.

Mycotoxin-reduction in fermented cereal food matrices is not exhaustively researched. It is therefore, necessary to screen probiotic microbial isolates to find those strains that have a definite potential to degrade aflatoxins during fermentation in food matrices (Ahlberg *et al*., 2015). Such mycotoxin-degrading species need to be fully compatible with the human GIT ecosystem (Nyanzi & Jooste, 2012). Despite the above assertion, lactic acid fermentation is the potential detoxification process for cereal-based beverages especially for African cereal beverages (Ahlberg *et al*., 2015; Nyamete, 2016; Nyanzi & Jooste, 2012; Waters *et al*., 2015). The mechanism of mycotoxin removal from fermented food matrices involves absorption of mycotoxins in their cell wall components as well as through active internalization and accumulation (Nyamete, 2016).

2.6.4 Fermentation and degradation of indigestible oligosaccharides

Indigestible oligosaccharides such as fructo-oligosaccharides and galacto-oligosaccharides are dietary fibres which are not degraded by the secretions of the human GIT especially in the small intestine (Mudgil & Barak, 2013). They perform similar physiological functions like other fibres (polysaccharides) such as softening stool consistency, increasing stool mass, enhance defecation frequency, improved transit time (laxative) that prevent constipation (Mudgil & Barak, 2013). Fermented oligosaccharides in the colon are also beneficial as they serve as food (prebiotic) for useful gut microflora (Derrien & van Hylckama Vlieg, 2015; Mudgil & Barak, 2013; Soro-Yao *et al*., 2014). Cereals and legumes contain indigestible oligosaccharides for instance; raffinose, stachyose and verbascose. These are sources of flatulence, diarrhea and indigestion (Hsieh & Ofori, 2007). The three sugars above have α-D-galactosidic bonds which are resistant to heat. However, some strains of LAB; such as *L. fermentum*, *L. plantarum*, *L. salivarius*, *L. brevis*, *L. buchneri* and *L. cellobiosus* possess galactosidase enzymes which can degrade these bonds. During fermentation, the microorganisms degrade these flatulence-causing and indigestible oligosaccharides into utilizable di-and mono-saccharides (Nyanzi & Jooste, 2012).

2.6.5 Fermentation and production of bacteriocins

Lactic Acid Bacteria isolated from fermented foods produce antimicrobial substances including bacteriocins which are protein in nature (Chelule *et al*., 2010; Todorov & Holzapfel, 2014). The presence of bacteriocin-producing LAB in fermented foods inhibit the growth of pathogens. Such pathogens include *Staphylococcus aureus, Bacillus cereus, Clostridium dificile* and *Listeria monocytogenes* (Tamang *et al*., 2016). The bactericidal actions of bacteriocins include the ability to form pores in the membrane of target bacteria, cellular DNA degradation, cleavage of 16S rDNA and halting of peptidoglycan synthesis (Todorov & Holzapfel, 2014). In such ways bacteriocins provide bactericidal and bacteriostatic effects in the GIT. The antimicrobial spectrum of bacteriocins is very narrow; as such nisin (from *Lactococcus lactis*) is the only one used as preservative in the food industry (Sahlin, 1999). *Lactobacillus spp*. produce varied characterized bacteriocins with potential benefits in fermented food preservation (Tamang *et al*., 2016).

2.7 Fermented cereal-based traditional beverages

Indigenous cereal-based beverages are consumed in many parts of the world; such as Southern Mexico (Atole – made from maize), Caucasus (Bagni-made from millet), Egypt (Bouza-made from wheat), Albania, Turkey, Bulgaria and Romania (Boza-made from wheat, millet, maize/other cereals), Indonesia (Brembali-made from rice), China, Korea, Japan, India, and Philippines (Blandino *et al*., 2003; Chaves-López *et al*., 2014; Nyanzi & Jooste, 2012). Traditionally, fermented cereal-based beverages (non-alcoholic and alcoholic) in many parts of Africa are prepared through spontaneous fermentation. The cereals involved include maize, millet, sorghum, finger millet, and millet malt (Enujiugha & Badejo, 2017; Mukisa *et al*., 2017). *Lactobacillus spp*. and yeasts are mainly involved in this kind of fermentation (Aka *et al*., 2014; Mugula *et al*., 2003b; Mugula *et al*., 2003a). Spontaneous fermentation involves two stages; lactic acid fermentation induced by a variety of environmental microorganisms and alcoholic fermentation initiated by dried yeast or a portion of previous brew (Olasupo *et al*., 2010). It improves the preservation, nutritional value and sensory characteristics of the beverages (Aloys & Angeline, 2009; Olasupo *et al*., 2010). Lactic acid fermentation produces non-alcoholic beverages whereas lactic acid and alcoholic fermentations give rise to alcoholic beverages (Kohajdová, 2016). Table 2 gives some examples of traditional African cereal-based beverages.

Table 2: Typical examples of African fermented cereal-based beverages

Beverage	Type of beverage	Country	Reference
Obushera	Non-alcoholic	Uganda	Franz *et al*. (2014)
Kanun-zaki	Non-alcoholic	Nigeria	Franz *et al*. (2014)
Pito	Alcoholic	Ghana, Nigeria	Blandino *et al*. (2003)
Busaa	Alcoholic	Kenya	Aka *et al*. (2014)
Mahewu	Non-alcoholic	South Africa	Blandino *et al*. (2003)
Tchapalo	Alcoholic	Côte d'Ivoire	Aka *et al*. (2014)
Umqombothi	Alcoholic	South Africa	Mokoena *et al*. (2016)
Borde	Non-alcoholic	Ethiopia	Enujiugha and Badejo (2017)
Gowé	Non-alcoholic	Benin	Aka *et al*. (2014)
Togwa	Non-alcoholic	Tanzania	Mugula *et al*. (2003)
Tchoukoutou	Alcoholic	Togo, Benin	Aka *et al*. (2014)
Mangisi	Non-alcoholic	Zimbabwe	Nyanzi and Jooste (2012)
Koko sour water	Non-alcoholic	Ghana	Aka *et al*. (2014)

2.8 Fermented cereal-based probiotic beverages

These are fermented probiotic beverages with cereals used as probiotic carriers (Arslan & Erbas, 2015; Enujiugha & Badejo, 2017). Recently, a wide variety of traditional non-dairy fermented beverages are produced globally, with non-alcoholic beverages made from cereals dominating (Enujiugha & Badejo, 2017; Waters *et al*., 2015). It has been reported that cereal and cereal component-based foods offer opportunities to include probiotics, prebiotics, and fiber in human diet (Lamsal & Faubion, 2009; Enujiugha & Badejo, 2017). Cereals contain water-soluble fibres (β-glucan and arabinoxylan), oligosaccharides (galacto- and fructo-oligosaccharides) and resistant starch, as such fulfill the prebiotic concept (Enujiugha & Badejo, 2017). Table 3 elucidates known probiotics as isolated from their substrates.

Table 3: Examples of probiotic isolates from known cereal-based foods

SN	Bacteria/Fungi isolates	Substrate	Food native region
1.	*Lactobacillus spp.* (**probiotic**)	Cereal-based foods and beverages (e.g., Togwa)	**Africa:** Tanzania, Zimbabwe, Ghana, S. Africa, Nigeria, Togo, Benin, Uganda
2.	*Lactobacillus paracasei* (**probiotic**)	Fermented uji	**Africa:** Tanzania, Kenya, Uganda
3.	*Lactobacillus acidophilus* (**probiotic**)	Sorghum-based products	**Africa:** Uganda, Tanzania, Zambia
4.	*Saccharomyces cerevisiae, Leuconostoc mesenteroides subspp. Mesenteroides, Leuconostoc confusus* (mixed culture)-(**probiotic**)	Millet, maize, wheat, rye, rice + other cereals + sugar (**Boza**)	**Europe (Balkan):** Bulgaria, Romania, Albania, Turkey
5.	*Lactobacillus casei, Leuconostoc mesenteroides, Saccharomyces cerevisiae* (mixed culture)-(**probiotic**)	Rye and barley malt, rye flour, stale rye bread, and sucrose (**Kvass**)	**Eastern Europe:** Czech Rep, Poland, Croatia, Georgia, Hungary

Nyanzi and Jooste (2012)

2.9 Cereals as food matrix for production of African beverages

The major cereal grains used for making African non-alcoholic and alcoholic cereal-based beverages are sorghum (*Sorghum bicolor* (L.) *Moench*), pearl millet (*Pennisetum glaucum* (L.)), finger millet (*Eleusine coracana*) and maize (*Zea mays* (L.)) (McFarland, 2015; Mugula *et al*., 2003a). Nyanzi and Jooste (2012) and Aka *et al*. (2014) have dealt extensively with the traditional African cereal-based beverages by indicating the raw materials, local names, their locations, either non-alcoholic or alcoholic beverages, and the preparation methods (technologies). Whole grain cereals and cereal components provide an option of having probiotic vehicles with double-advantage of giving healthful bioactive components and fibers (Enujiugha & Badejo, 2017; Lamsal & Faubion, 2009; Waters *et al*., 2015). Such components include non-digestible carbohydrates, soluble fibre and phytochemicals such as phytoestrogens, antioxidants, phenolic compounds, phytic acids and terpenes (Lamsal & Faubion, 2009; Waters *et al*., 2015).

Despite being the main source of dietary nutrients globally, cereal grains lack some basic food components such as amino acids (Amadou., 2011; Marsh *et al*., 2014). Fermentation may improve the nutritional value, sensory attributes and functional qualities of cereals (Amadou., 2011; Navarrete-Bolaños, 2012).

2.9.1 Microbes associated with spontaneous fermentation of traditional cereal-based beverages

Production of African fermented cereal beverages involves competition among various species and strains of microbes and those best suited for the environment will multiply rapidly and dominate certain stages of the process (Aka *et al*., 2014; Oyedeji *et al*., 2013). Lactic acid bacteria are involved in lactic fermentation whereas yeasts are involved in alcoholic fermentation (Marco *et al*., 2017; Todorov & Holzapfel, 2014). Lactic acid fermentation induces advantageous characteristics observed in fermented foods. A number of studies have shown that LAB and yeasts are predominant in these beverages (Aka *et al*., 2014; Enujiugha & Badejo, 2017; Guyot, 2012; Mugula *et al*., 2003b; Oyedeji *et al*., 2013). However, majority of microbes isolated from fermented cereal beverages are LAB (Kohajdová, 2016; Mokoena *et al*., 2016; Oyedeji *et al*., 2013). In the mash prior to spontaneous fermentation LAB present include *Lactobacillus, Pediococcus, Leuconostoc* and *Enterococcus*. In traditional sorghum beer the microbes normally found belong to the genus *Lactobacillus, Leuconostoc, Lactococcus, Enterococcus* and *Streptococcus*. Typical examples are *L. plantarum, L. brevis, L. casei, L. sakei, L. acidophilus, L. delbrueckii, L. fermentum, Lactococcus lactis subsp lactis, Lactococcus raffinolactis,* and *Leuconostoc mesenteroides subsp mesenteroides*. The above results are based on conventional bacteriological techniques. Species or strains of *Lactobacillus* are mostly heterofermentative (Hammes & Hertel, 2009). It has also been reported by Enujiugha and Badejo (2017) that *L. fermentum*, *L. plantarum, Pediococcus pentosaceus, Weisella confusa,* and *L. rhamnosus* have been isolated from a traditional fermented maize porridge (sweetened beverage) in Kenya known as Ikii.

Yeasts of the genus *Saccharomyces* are predominant in alcoholic fermentation (Aka *et al*., 2014; Ray, 2004; Walker & Stewart, 2016). However, some researchers using molecular biology techniques have indicated that strains of *S. cerevisiae* exclusively predominate fermentation of sorghum-based African beverages (Lara-Hidalgo *et al*., 2017). Despite their importance in food fermentations; only two yeast species *S. cerevisiae,* and *S. cerevisiae subsp. boulardii* have been found to be probiotic (Gil-Rodríguez *et al*., 2015; Lara-Hidalgo *et al*., 2017).

2.10 Why African fermented cereal-based beverages are produced?

The beverages are produced through fermentation of germinated cereals such as maize, millet and sorghum. In Africa, some importance is attached to these beverages. They are important to individuals, families and the society as a whole. Socially, when served; they show a gesture of hospitality, friendliness and also to strengthen amicable relationships between individuals (Aka *et al*., 2014). They are also consumed in farm work, ceremonies such as marriage and funerals and as supplement food; weaning food for babies (health giving drinks). The beverages also improve lactation in nourishing mothers and prevent coronary diseases and cancer. They are consumed by all social classes. Non-alcoholic beverages are guzzled by all age groups; infants, children, pregnant women, the old and sick people. Alcoholic beverages are mainly consumed by men (Aka *et al*., 2014; Kalui *et al*., 2010). The African fermented cereal beverages are also consumed as food or drink. They provide energy in the diet (starch, sugars, proteins) and valuable nutrients such as B-group vitamins; thiamine, niacin and riboflavin as well as vitamin C (particularly in sorghum beverages). They are also sources of minerals like Fe, Ca, K, Mg, Mn, P and Cu (Kohajdová & Karovičová, 2007). Economically, sale of fermented cereal beverages provide income to households particularly women.

Fermentation improves palatability, aroma, flavor and taste to cereal beverages as a result of production of organic acids and volatile compounds such as lactic acid, acetic acid, propionic acid and acetaldehyde during the process (Kohajdová & Karovičová, 2007; Sahlin, 1999; Stepanek, 2015). It has been reported that despite fermenting the cereal gruel during Togwa production; LAB participate in proteolytic activities to give free amino acids and smaller peptides which are utilized by them to produce metabolites (Mugula *et al*., 2003c). Most of the African fermented cereal-based beverages are characterized by their colour, taste, odour, and flavour, consistency, shelf-life and alcohol content. The colour of the beverages can be pinkish-brown, whitish-grey to brown, light brown, creamy or milky (colour of raw materials). The beverages have sweet-sour taste, sweet-sour odours and flavours as well as being opaque with suspended microbes and solid particles. Furthermore, they lack or have low level of alcohol and their shelf-life is shorter (Aka *et al*., 2014; Aloys & Angeline 2009; Kitabatake *et al*., 2003; Oyedeji *et al*., 2013; Mukisa *et al*., 2016, 2017; Mokoena *et al*., 2016). In most cases consumption of traditional African fermented beverages is done in the fermenting state; as such organoleptic qualities vary.

African fermented cereal beverages have prolonged shelf-life because fermentation produces organic acids such as butyric, propionic, lactic as well as acetic acids which lower their pH to below 4 and consequently inducing inhibitory effects to growth and proliferation of both spoilage and pathogenic microbes (Aka *et al*., 2014; Kohajdová & Karovičová, 2007; Mugula *et al*., 2003c; Oyedeji *et al*., 2013). Anti-microbial metabolites (e.g., carbon dioxide, ethanol, hydrogen peroxide) produced by LAB also enhance the shelf-life of beverages though their shelf-life remains limited (less than or equal to 3 or 5 days). Limited shelf-life of such beverages poses a challenge to producers.

Traditional fermented cereal-based beverages have preventive and curative properties for many known diseases. Some researchers have indicated that useful bacteria in fermented foods assist the digestive system in food assimilation and production of B-vitamins in the beverages (Kohajdová & Karovičová, 2007). The B-vitamins produced by LAB include niacin (B_3), pantothenic acid (B_5), folic acid (B_9), B_1, B_2, B_6 and B_{12}. These vitamins are important co-factors in some metabolic reactions in the human body (Fernandesa *et al*., 2018). The beverages have anti-inflammatory, anti-diarrheal, anti-bacterial, anti-tumor, anti-spasmodic, laxative, anti-malarial, anti-hemorrhoid, anti-oxidant properties (Iqbal *et al*., 2014; Murevanhema & Jideani, 2013; Nyanzi & Jooste, 2012).

2.11 African fermented cereal-based beverages vis-à-vis starter cultures

Natural fermentation is applied in making the traditional fermented cereal-based beverages. Naturally occurring microorganisms can be from the raw materials, equipment used or the environment. This procedure has been handed down from one generation to another. Since starter cultures are not used; such fermentations are uncontrolled and thus the quality, safety and stability of the products are compromised (Aloys & Angeline, 2009; Guyot, 2012).

Investigations made have so far revealed that LAB and yeasts are the major groups of microbes in fermented cereal-based beverages (Aka *et al*., 2014; Guyot, 2012; Mugula *et al*., 2003c). While the presence of yeasts favours the growth of bacteria through provision of growth factors, multiplication of yeasts in the beverages is favored by the acidic environment induced by LAB (Kohajdová & Karovičová, 2007; Mugula *et al*., 2003b; Mugula *et al*., 2003a). This is the main reason of their predominance in the beverages as the growth of other microbes is inhibited by the acid medium as well as the metabolites produced by the two groups of

microorganisms. The metabolites produced by the LAB and yeasts impart flavor and taste to the beverages (Aka *et al*., 2014; Mugula *et al*., 2003c).

Not all fermentative *Lactobacillus spp.* identified in African cereal-based beverages are probiotic, but the greater part of the microbes exhibit probiotic qualities (Navarrete-Bolaños, 2012). *Lactobacillus acidophilus* is the LAB species which predominates in the intestinal tract of healthy humans and most often probiotic products contain this microbe (Enujiugha & Badejo, 2017; Marsh *et al*., 2014). However, probiotic organisms are invariably not suitable as starter culture because of the difference in the environment within the GIT and that in the food matrix. To overcome this problem, usually a help culture is added to a probiotic preparation (Enujiugha & Badejo, 2017; Hassan *et al.,* 2012). While preparing fermented product having probiotics; it is recommended to carry out the fermentation at a temperature range of 37 – 40 °C since most probiotic strains increase exponentially in that temperature range (Marshall & Mejia, 2012).

Successful use of various species of LAB and yeasts as pure starter cultures and co-cultures to produce traditional fermented cereal products has been reported by Aka *et al*. (2014). Some of the drinks in which the co-culture has been used are reported to be potential probiotic foods when hygienically produced. Such drinks are Pito (Nigeria, Ghana) and opaque sorghum beer Tchoukoutou (Benin, Togo). It has also been observed that production process of Enturire; an alcoholic sorghum-and honey-based beverage produced in Uganda can be modified and shortened by using pure starter cultures of LAB and yeasts instead of spontaneous fermentation (Mukisa *et al*., 2016). Based on the above studies; Africa needs to develop pure starter cultures which contain efficacious microbial strain that can lead to control and optimization of the fermentation process so that beverages produced are of high organoleptic quality, safety and microbial stability. It is thus obvious that selection of an appropriate strain or strains for a particular fermented product is paramount as a first step in a fermentation process which is controllable, predictable and efficient (Navarrete-Bolaños, 2012).

2.12 Quality and safety of African fermented cereal-based beverages

Use of cereals such as maize, millet and sorghum which contain antinutritional factors (phytates, oxalates, tannins) impede the bioavailability of vitamins, proteins and minerals and may lead to anaemia, and nutritional deficiency conditions like malnutrition and stunted growth. The impairment of absorption of minerals such as Fe, Ca, Mg, Mo, Zn is due to

complex formation with tannins and phytates (Kohajdová & Karovičová, 2007). Some African fermented cereal-based beverages like Burukutu (Northen Nigeria), Malwa (Uganda) and Tchoukoutou (Benin, Togo) were reported to contain tannins and phytates (Sundarraj *et al*., 2018; Anukam & Reid, 2009; Nout, 2009). Some African fermented cereal beverages are prone to heavy metal (Pb, Hg, As, Cd) contamination from the environment (Aka *et al*., 2014); however, this is not always the case and may probably depend on the location where the production is done and possibility of exposure from contaminated soil or water. In human, heavy metals cause food poisoning and other toxic effects; e.g., renal toxicity (Food Safety Authority of Ireland, 2009).

Most of the African fermented cereal-based beverages are prepared at homes. Preparations in unhygienic environment make the beverages susceptible to contamination by microflora (Blandino *et al*., 2003b). Contamination in beverages mainly caused by humans, utensils, raw materials, processing devices, environment, sewage, rodents, poor storage and handling conditions (Adams & Moss, 2008). *Aspergillus flavus, S. aureus, A. niger, E. coli, Bacillus spp., Proteus spp., Streptococcus spp., Rhizopus stolonifer* are pathogens contaminating the African cereal beverages (Aka *et al*., 2014).

2.13 Beverages as vehicles for delivery of probiotics

Literature indicates several beverage foods which are employed/potential as probiotics carriers. These include dairy products particularly yoghurts, fermented cereal beverages, fruit juices and liquid dietary supplements (Bansal *et al*., 2015; Enujiugha & Badejo, 2017; Mukisa, 2016). This section describes cereals (fermented cereal beverages) as the emerging probiotics carriers particularly for African countries.

2.13.1 Fermented cereal-based probiotic beverages as probiotics delivery vehicles

African fermented cereal-based beverages with potential probiotics have health benefits related to probiotics (Arslan & Erbas, 2015; Kalui *et al*., 2010). Development of fermented cereal based probiotic beverages is a welcome initiative for provision of probiotic products to the majority of people. Consequently, millions of people will derive health benefits from the health-giving microorganisms. The WHO in 1994 indicated that probiotics are going to be the immune defense system of choice after ever increasing antibiotic resistance of existing antibiotic drugs (Kalui *et al*., 2010).

The need for alternative sources of probiotics carriers to meet food preferences and demands of certain segments of the consumer market, have led to more researches around the world on various food matrices like fruit juices, vegetables and cereals (Bansal *et al*., 2015; Gupta & Bajaj, 2016; Nyanzi & Jooste, 2012). Fermented cereal-based probiotic beverages are very few in the consumer market compared to probiotic milk or milk products (Angelov *et al*., 2006). Cereals make up the larger portion of staple diet for majority of people in Africa and other developing countries and thus there is justification for more researches on the use of spontaneously fermented cereal beverages as probiotics carriers. Moreover, it is known that 60% of the diet in developing countries is prepared from fermented cereals (Waters *et al*., 2015).

A handful of studies have been done on the possibility of using African traditional fermented cereal-based beverages as probiotics carriers (Angelov *et al*., 2006; Kalui *et al*., 2010). In one study, fermented rice or millet probiotic beverage were made using *Streptococcus thermophilus, L. acidophilus* and *bifidobacterium BB-12*; collectively referred to as ABT-2 starter culture and fortified with 10% each of sesame and pumpkin milk. Honey (5%) was also added before inoculation (Hassan *et al*., 2012). At the end of the 16 hours fermentation time; the viable cell count was about 4.3 x 10^9 cfu/mL. Fortification changed acidity and probiotic bacteria counts. However, during storage of the beverages at 4 °C; slight changes in the viable counts, acidity and pH were observed. Moreover, the organoleptic characteristics were improved. The shelf life of the two beverages was estimated to be 15 days at +4 °C during which the pH and acidity of each beverage remained above 4 and lower than 1%. Within the shelf-life period, the probiotic bacterial count of the ABT-2 starter culture stayed at 8 log cfu/mL.

Traditional African fermented millet-based beverages with probiotic potential by having some probiotics in them include Koko (West Africa), Mangisi (Zimbabwe and Uganda), Uji (East Africa), Burukutu and Pito (Togo, Benin), Kunun-zaki (Nigeria, Niger, Tchad), Ogi (West Africa), Ben-saalga (Burkina Faso), Togwa (Tanzania) (Enujiugha & Badejo, 2017; Franz *et al*., 2014; Amadou, 2011). On the other hand, some traditional fermented cereal-based beverages are produced from sorghum malt. In one study, mixed starter cultures of LAB and yeasts used in the production of Obushera; a traditional sorghum malt fermented beverage showed that in addition to producing lactic acid during fermentation, LAB produce flavour compounds as well (Mukisa *et al*., 2017). The flavour compounds include diacetyl, acetate,

ethanol, acetaldehyde, acetone and acetoin. The key flavor compounds added by yeasts are acetaldehyde, methyl aldehydes, ethanol and methyl alcohol (Mukisa *et al*., 2017). Obushera produced was potentially probiotic as it contained probiotic microorganisms.

Studies on development of cereal-based probiotic beverages have not only been done using cereals which are staple foods in Africa but also cereals available in other parts of the world. Angelov *et al*. (2006) reported preparation of an oat-based probiotic beverage through an 8 h controlled fermentation at 37 °C. The beverage was inoculated with *L. plantarum* as a starter culture and at the end of the fermentation time, it was observed that the product had viable cell count of 7.5 x 10^{10} cfu/mL and pH of 4.0 - 4.5. A probiotic fermented oat-flour beverage (PFOF) was developed from a suspension of oat flour in distilled water. The suspension was inoculated with culture of *L. plantarum* and fermented at 37 °C for 72 h resulting to a probiotic beverage with viable cell count of 10^{14} cfu/mL (Gupta & Bajaj, 2017). This shows that the oat-flour matrix supports the growth of probiotic bacteria. Salmerón *et al*. (2015) reported development of three cereal-based probiotic beverages from three cereal substrates; malt, barley, and oat after 10 h of fermentation at 37 °C by means of mono-culture strains of *L. plantarum, L. acidophilus*, and *L. reuteri*. The developed cereal beverages had viable cell counts of 7.8 – 8.2 log cfu/mL. Other studies with positive results on development of fermented cereal-based probiotic beverages include those by Ogunremi *et al*. (2015) and Di Stefano *et al*. (2017).

2.13.2 Benefits of the cereal-based probiotic beverages

They are considered as alternative delivery vehicles for probiotics to cater for those people in Africa and elsewhere who can’t consume milk or milk products because of religious beliefs, cultural food taboos, high cholesterol content, allergenic to milk and being vegetarian or vegan (Gupta & Bajaj, 2016; Nyanzi & Jooste, 2012). They provide health benefits to majority of people especially rural people through cheaper probiotics from cheaper sources of food. Fermentation of cereals reduces/removes antinutritional factors and mycotoxins from them and thus provides essential minerals and vitamins to beverage consumers (Nyamete *et al*., 2016; Nyanzi & Jooste, 2012). The probiotics they contain produce metabolites such as organic acids and bacteriocins which act as bio-preservatives for the beverages. Since the cereals used for production of the fermented cereal-based probiotic beverages are customarily staple foods in Africa; they can easily be sourced. Of great importance is the fact that heavy dependency on milk and milk products as probiotic carriers will be minimized and this will lead to increased

availability of cereal-based foods. The only challenge to the multitude of the benefits above is the fact that fermented cereal-based probiotic beverages are strain specific and thus careful screening and testing need to be done to obtain a specific, efficacious and safe probiotic strain for a given cereal substrate. Table 4 summarizes benefits of probiotic cereal-based beverages as compared to diary based probiotic products.

Table 4: Probiotic fermented cereal beverages as compared to diary-based probiotics

Probiotic fermented cereal beverages	Diary based probiotics	Reference
Product with more nutrients; e.g., vitamin Bs (B_1, B_2, B_3, B_5, B_6, B_9 and B_{12}), fibre especially indigestible fructo- and galacto-oligosaccharides which are used as prebiotics in the gut, digested carbohydrates (CHOs); i.e., utilizable di and monosaccharides, proteins and minerals (Fe, Zn, Mg, Ca, Mn, Mo) that are released by enzymatic degradation of cereals by LAB	Nutrients available, not as much as cereal based (e.g., lack of fiber, lack Fe, Zn, Mn, Mo, lack some B vitamins (B1, B2, B3, B5, B9 etc.)	Blandino *et al*. (2003) Kohajdová and Karovičová (2007) Enujiugha and Badejo (2017)
Consumed by people of all age groups and health status as they are free of disorders caused by milk and milk products	Vegetarians, vegans, lactose intolerant and allergic people, people with milk taboo, some believers, hypercholesterolemic can't consume the products	Nyanzi and Jooste (2012) Vasudha and Mishra (2013) Aka *et al*. (2014)
Low production cost as the cereals are staple food locally available and beverages produced at household level or small-scale enterprises	High production cost as milk production involves a lot of processes e.g., raising dairy cattle, milking, sterilization, homogenization, cooling, packaging, cold chain maintenance	Franz *et al*. (2014)
Easily acceptable by local Africans as they make use of staple foods; maize, millet, sorghum to which they are accustomed	Milk and milk products may not be a local solution to nutrition and health maintenance as they are not part of the daily diet and can be too expensive for rural people in Africa	Kalui *et al*. (2010) Franz *et al*. (2014)
Fermented substrates (cereals) are ubiquitous in the world (73% of cultivated area) and provide more than 60% of the world food production	Milk is not available in all African communities; milk fermentation is mainly practiced in Northern West Africa, Northern Africa, the Sahara, the Savannah and hills and hill foots of East Africa	Kohajdová and Karovičová (2007) and Todorov and Holzapfel (2014) Franz *et al*. (2014)
Several alternative probiotic carriers are available (maize, millet, sorghum, oats, barley)	Dependency on milk and milk products as the only probiotic carriers	Enujiugha and Badejo (2017)

Probiotic fermented cereal beverages	Diary based probiotics	Reference
Probiotic strains are specific to a given substrate based on presence of necessary growth factors such as Carbon source, nucleic acids, vitamin B-group, amino acids and minerals	Many probiotics grow on diary media as they have nearly all necessary growth factors	Nyanzi and Jooste (2012) McFarland (2015) Aka *et al*. (2014)
Prevent and cure known and life style diseases e.g., diarrhea, Cardiovascular disorders, allergy, lactose intolerance, hypercholesterolemia, constipation, urogenital tract infection, high blood pressure, cancer	Prevent and cure known and life style diseases e.g., diarrhea, Cardiovascular disorders, allergy, lactose intolerance, hypercholesterolemia, constipation, urogenital tract infection, high blood pressure, cancer	Kechagia *et al*. (2013) Hassan *et al*. (2012)

2.14 Food matrices, quality and functionality of cereal-based probiotic beverages

Scarce knowledge is available regarding the effect of food matrix and product formulation on probiotic functionality. Probiotic culture functionality; survival, physiology and efficacy may be affected by type of food format (Kumar *et al*., 2015). Preparation of a functional probiotic food requires selection of a suitable food carrier and maintenance of viability and sensory characteristics which play a key role in making the product succeed in the market (Dey, 2018; Rivera-Espinoza *et al*., 2010; Kumar *et al*., 2015). Viability of probiotic cells during production of probiotic beverages is greatly reduced by technological conditions such as heat, mechanical damage and cell injury due to osmotic stress (Dey, 2018; Kumar *et al*., 2015).

The probiotic quality and functionality of the fermented cereal-based beverage lies in its ability to have viable probiotic bacteria in the range from $10^6 – 10^7$ cfu/g and promote human health at the target organ/site (Enujiugha & Badejo, 2017; Hassan *et al*., 2012; Nyanzi & Jooste, 2012; Ranadheera *et al*., 2017). Thus, when probiotic cultures are ingested; they should survive in sufficient numbers when transiting through the gastrointestinal tract in order to elicit their effects at target sites (Enujiugha & Badejo, 2017; Panghal *et al*., 2018; Rivera-Espinoza *et al*., 2010). Some of the recent techniques used to improve the survival of probiotic strains during food processing and when ingested by human include encapsulation in prebiotics, genetic technique as well as a traditional way of increasing the inoculum size to pre-determined higher levels of bacteria (Dey, 2018; Enujiugha & Badejo, 2017). Some researchers have suggested that *in vitro* or *in vivo* survival of probiotics is highly influenced by the food carrier (Kalui *et al*., 2010).

The survival of probiotics during processing and long term storage heavily relies on the type of food matrix, rate of moisture content and cell condition (Kumar *et al*., 2015). Lactic acid bacteria grow well in cereals and this shows that probiotic fermented beverage with definite and consistent properties can be made using a human-derived probiotic strain in a cereal substrate under controlled conditions (Charalampopoulos *et al*., 2002). However, to produce such a product several considerations have to be made. These include composition and processing of the cereal grains, starter culture growth ability and productivity, probiotics stability during storage, sensory properties, and nutritional value of the end product (Charalampopoulos *et al*., 2002; Dey, 2018). The ability to survive the acidic conditions of the ultimate fermented product and the harsh conditions of the GIT form a main factor in the selection of requisite probiotic bacteria. Furthermore, physical and chemical properties of the delivery media (food); e.g., buffering capacity and pH affect the survival of the probiotic bacteria during transit across the GIT (Charalampopoulos *et al*., 2002; Kalui *et al*., 2010).

The ability of probiotics to survive during preparation and storage of probiotic product is a main factor in producing such foods. Microbial growth and survival is essentially determined by ability to adapt to changing environments (Enujiugha & Badejo, 2017; Kumari *et al*., 2015). It is a requirement that a probiotic has to show high survival rates in downstream processes, in food products during storage, in the upper Gastrointestinal tract, at the site of action as well as high activity in the gut environment (Kumari *et al*., 2015). Storage of cereal products (beverages) at room temperature may affect their probiotic stability (Enujiugha & Badejo, 2017). Encapsulation technology can be used to guarantee viability and stability of probiotic cultures (Charalampopoulos *et al*., 2002). However, some studies at ambient temperature have indicated that viability and stability of probiotics can be achieved. A probiotic water-based millet beverage (8% pre-treated millet in water, 5% sucrose) was prepared through controlled fermentation with co-culture of *L. rhamnosus* GR-1 + *Streptococcus thermophilus* C106 and maintained at ambient temperature for 5 days with viability of 10^9 cfu/mL and pH 3.9 (Di Stefano *et al*., 2017). Similarly, Gupta and Bajaj (2017) have depicted that storage of a probiotic oat beverage at ambient temperature ensured stability of the product for more than three weeks with viability of 10^6 cfu/mL of the *L. plantarum* culture. It has also been reported that probiotic bacteria can maintain their viability and stability even at lower storage temperatures such as refrigeration temperatures. Di Stefano *et al*. (2017) prepared a water-based millet product (10% millet in water, 5% sucrose) that remained stable at 4 °C for 56 days with viability of more than 10^8 cfu/mL. In another similar study, Gupta and Bajaj (2017)

developed a probiotic beverage by fermenting oat flour with *L. plantarum* and the resultant probiotic fermented oat flour beverage (PFOF) was stable at 4 °C with viability more than 10^6 cfu/mL. The preceding studies vividly show that the viability and stability of probiotic cereal-based beverages can be achieved and maintained at both ambient and refrigerated storage with careful selection of a fermenting strain or strains of probiotic bacteria.

It is important to perform viable counts for stored fermented probiotic foods on economic, technological and efficacious reasons. The quality of the probiotic products is enhanced by the packaging material used and the storage conditions under which they are reserved (Kumari *et al*., 2015). In the probiotic product; the probiotic culture has to contribute to good sensory characteristics of the product. In order to have fermented cereal product with desired organoleptic properties; selection of an appropriate strain is mandatory in order to achieve distribution of the requisite metabolic end products (Charalampopoulos *et al*., 2002; Kumari *et al*., 2015). As such it is normal to use a co-culture of probiotic bacteria in order to achieve fermentation of a particular product (Enujiugha & Badejo, 2017; Kumari *et al*., 2015). Understanding the biochemical pathways for production of specific flavours assists in choosing the right starter. End product distribution of lactic acid fermentation is also determined by substrate composition and environmental conditions (e.g. temperature, pH) (Charalampopoulos *et al*., 2002; Kohajdová & Karovičová, 2007).

Generally, African traditional fermented beverages are considered synbiotics because they contain both indigestible polysaccharides as well as the LAB which ferment the substrate (Lamsal & Faubion, 2009; Mokoena *et al*., 2016). Though Africa has a rich diversity of fermented beverages; their utilization as potential sources of novel probiotics is still a challenge to African researchers and producers (Mokoena *et al*., 2016; Nyanzi & Jooste, 2012). As such there is a need to increase research capabilities on how best to isolate, identify and screen potential probiotics which are safe and efficacious from local beverages for use in fermented cereal-based probiotic beverages.

2.15 Importance of probiotic cereal-based beverages

Probiotics consumption have numerous benefits to human health such as boosting lactose tolerance, prevent constipation, prevention of allergies, improve balance of intestinal microflora, reduction of intestinal pH, reduction of cholesterol and risk of colorectal cancer, prevention and treatment of acute diarrhea and urogenital tract infections, inhibition of the

helicobacter pyroli infection, modulation of the immune response, replenishing of intestinal microflora after treating certain diseases, release of B-group vitamins, decrease ammonia and other toxic substances and enhance functioning of the intestines (Charalampopoulos *et al*., 2002; Enujiugha & Badejo, 2017; Kechagia *et al*., 2013; Vasudha & Mishra, 2013). Furthermore, treatment with probiotics shorten the duration of infections as well as slows down susceptibility of host to pathogens (Kumar *et al*., 2015).

In sub-Saharan Africa 37% of childhood deaths is attributed to diarrhea. Such deaths could be cut down by using fermented probiotic foods like fermented cereal-based probiotic beverages (when their potentiality as probiotic carriers is fully put into use) to improve the health of the children (Anukam & Reid, 2009; Franz *et al*., 2014). Evidence exists which shows that some probiotic strains are efficacious in preventing and treating acute diarrhea (Aka *et al*., 2014; Kalui *et al*., 2010). One study has indicated that consumption of fermented maize gruel is able to mitigate prevalence of fecal enteric bacteria (*Shigella, Salmonella, E. coli*) in young children (Kalui *et al*., 2010). Fermentation; a process used in making cereal-based beverages breaks down and detoxifies the cereal raw materials thus raising the uptake of macro- and micro-nutrients as such reducing malnutrition in Africa (Franz *et al*., 2014; Nyamete, 2016; Nyanzi & Jooste, 2012).

In African countries and other developing countries; cereals occupy a larger share of the staple diets and this forms the basis for introducing probiotics in such foods particularly cereal beverages which are consumed regularly in social gatherings and other important community rituals. Such beverages in turn can provide health benefits including prevention and curing of diseases. Though traditional African fermented cereal beverages have proven probiotic potential; some research gaps have to be filled in. These include isolation of novel probiotics and development of stress-tolerant starters with proven probiotic attributes from the beverages; that are able to maintain their probiotic qualities and functionality while transiting the Gastrointestinal tract (GIT) to the target site in the colon.

2.16 Production processes of some cereal-based beverages in Tanzania

In many parts of sub-Saharan Africa; alcoholic and non-alcoholic beverages are made through saccharification of starch from cereals, flours of root crops and germinated cereals (malt). These drinks have long been in existence and are popular to date (Kitabatake *et al*., 2003). Beverages made from malt flour are the most original and traditional beverages (Kitabatake *et*

al., 2003). Alcoholic and non-alcoholic beverages in sub-Saharan Africa are turbid compared to modern alcoholic drinks as they are not filtered off sufficiently to remove the contaminants from the broth i.e. insoluble materials, remaining residues, bacteria, yeast and other contaminating substances (Kitabatake *et al*., 2003).

2.16.1 Togwa in Southern Tanzania

Non-alcoholic beverages are prepared from both cereal or tuber flour and malt flour and have been consumed for many years as refreshments, food for the sick, weaning food and energy drink for working farmers. Togwa; with a characteristic sweet and sour taste is one of such drinks and is mostly consumed in East Africa (Kitabatake *et al*., 2003). It is an intermediate product during the production of alcohol from malt flour and cereal or tuber crop flour (Kitabatake *et al*., 2003). In the southern part of Tanzania Togwa is predominantly regarded as a food for working people. In this area, Togwa is prepared according to the procedure given in Fig. 1. The ingredients used in making Togwa are maize flour, finger millet malt flour and water. They are used in pre-determined proportions.

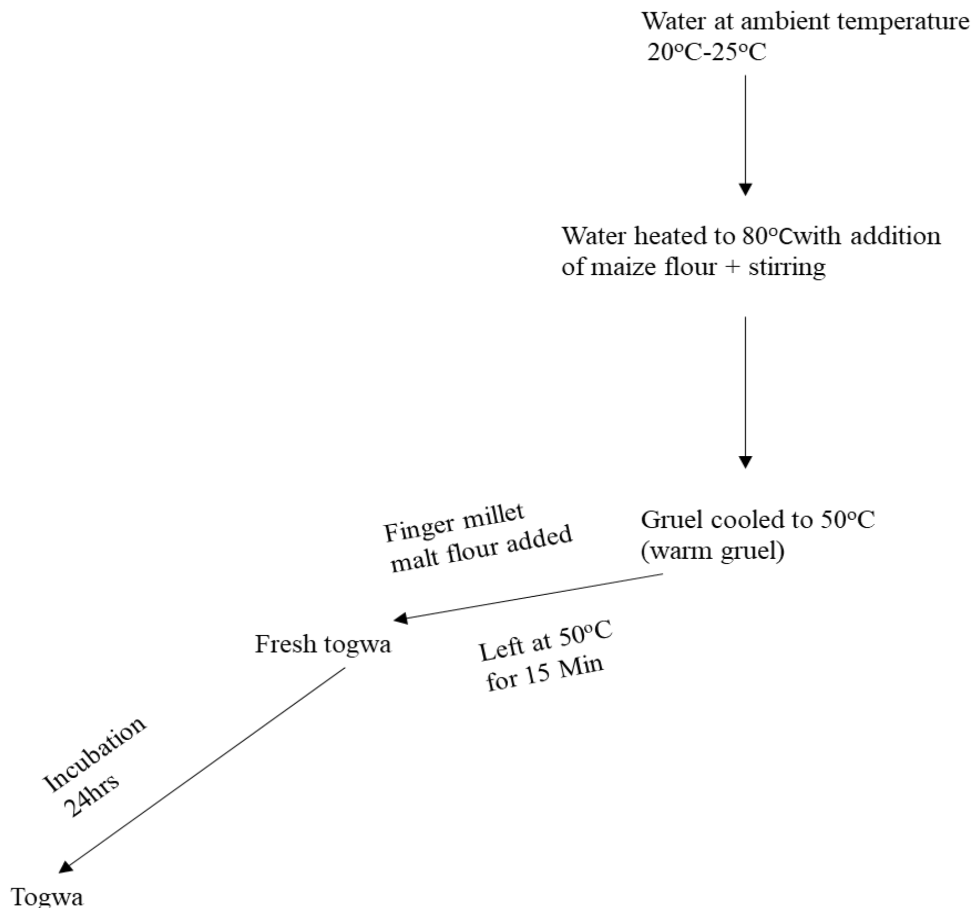

Figure 1: Flow diagram for preparation of Togwa in Southern Tanzania

Togwa is opaque and brownish in colour; the colour originating from the particles of hulls of finger millet and maize seeds. Kitabake *et al*. (2003) showed that fresh Togwa is sweet after 5 h to less than 25 h of incubation; however, the sourness increases after 48 h of incubation. In the Southern part of Tanzania, the beverage is always fit for consumption within 72 h after making it; otherwise, it gets spoilt. The generation of simple sugars and oligosaccharides such as glucose, maltose, maltotriose, triose imparts sweetness to the beverage while the fermentation of lactate to lactic acid gives it sourness.

2.16.2 Mbege in North Eastern Tanzania

Mbege is a renowned fermented cereal-based beverage in the North Eastern part of Tanzania. It is made from ripe bananas (Musa *spp*. locally known as *Mgomba* type, *Ndizi ng'ombe*, *Katani*, *Uganda*, *Kisukari*, *Malali*), finger millet (*Eleusine coracana*) flour, and water (Kubo & Kilasara, 2016). Pieces of *Msesewe* tree (*Rauvolfia caffra*) root or stem bark are added as additives and accelerate the fermentation of *Nyalu* (ripened banana fermented porridge) as well as increase the yield of ethanol (alcohol) during Mbege production (Kubo & Kilasara, 2016). Mbege is prepared in three important steps; first production of *Nyalu* followed by production of *Mso* (a sweet gruel prepared from cooked finger millet malt flour) and finally mixing of the two gruels to get Mbege as a final product after fermentation. *Nyalu* is used as a source of yeasts whereas *Mso* is used as a source of fermentable sugars (Kubo & Kilasara, 2016).

Kubo and Kilasara (2016) observed that production of Mbege starts with the preparation of *Nyalu*. *Nyalu* is prepared by mixing 36 L of ripened bananas with an equal volume of water in a drum. The mixture is cooked on a flame till the lumps are wholly broken down, and it is then poured into a wooden barrel and left to stand at ambient temperature uncovered for 10-12 days undergoing fermentation. *Msesewe* powder is usually added after cooking the bananas. After fermentation, the mixture is filtered off by means of a jute bag and the filtrate obtained is *Nyalu*.

When *Nyalu* is ready, *Mso* preparation starts. The *Mso* is prepared by mixing 35 L of water in a metal barrel with 5 L of finger millet malt flour and heating the mixture for a few minutes. Then a 15 L portion of warm, thin mixture is removed and set aside for use in thinning out the main mixture to avoid drop in temperature. Following that, additional 15 L of millet malt flour is added into the main mixture which is strongly stirred with the aid of a wooden spoon and thinning using the thin mixture continuing irregularly. The *Mso* is cooked to a temperature range of 50 - 70 °C to avoid denaturing of the enzyme α-amylase which is important for

hydrolyzing complex insoluble polysaccharides into simple fermentable sugars. The final cooked mixture is *Mso* which is then placed in containers for cooling. It is a brown thick porridge which tastes sweet (Kubo & Kilasara, 2016).

Mbege is obtained by mixing *Nyalu* and *Mso* in a wooden barrel and left to ferment for 12-48 hours. During this period the alcohol content of Mbege increases from 3.2 to 4.0 %, °Brix decreases from 7.5 to 6.0 and pH remains at 4.2 (Kubo & Kilasara, 2016). Mbege is a reddish alcoholic beverage with a moderate sour taste. It is drunk by almost all age groups and its shelf life is at most two days (Kubo & Kilasara, 2016).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sampling and sample collection sites

Collection of samples of each cereal-based beverage (Kimpumu, Kindi, Togwa, and Mbege) was done from the local producers in Mbeya, Morogoro and Kilimanjaro regions. Mbeya and Morogoro regions were chosen based on previous studies on Togwa (Kitabatake *et al*., 2003; Mugula *et al*., 2003a) and existence of a large number of households of Kindi and Kimpumu producers in Mbeya. Kilimanjaro was chosen following the study by Kubo and Kilasara and being the main hub for Mbege producers. One district per region was purposively selected from which one village was identified based on accessibility of the local beverage. From each of the villages, quadruplicate samples (each 100 mL) from different producers were aseptically collected by means of a sterile syringe for each type of beverage. Glass bottles with the samples were labeled and kept in a cool box for transportation to the laboratory for impending analysis. In the lab, pH of each sample was measured twofold and the average recorded. Samples were then stored at 4 °C until needed for analysis. Plate 1 depicts the cereal-based beverages in containers as observed in the field and Fig. 2 shows sample collection sites.

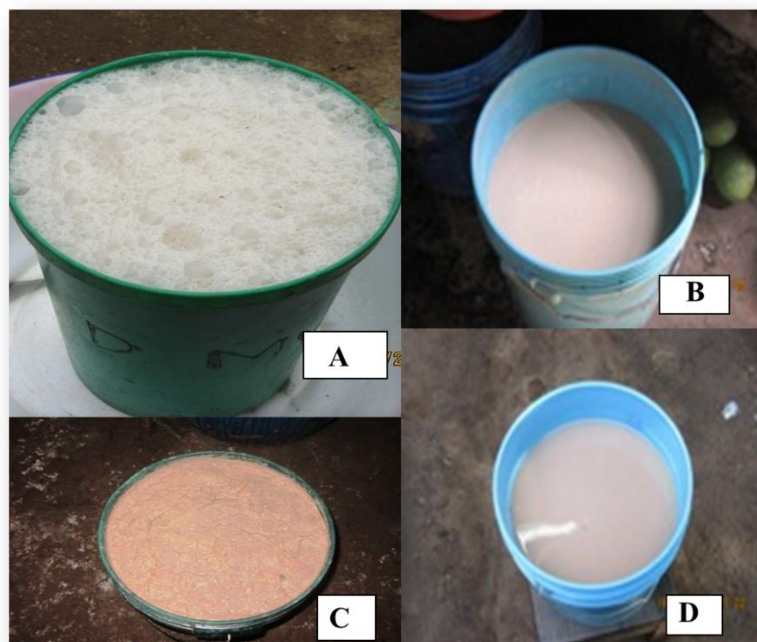

Plate 1: Sampled beverages: A- Kindi; B- Kimpumu; C- Mbege; D-Togwa

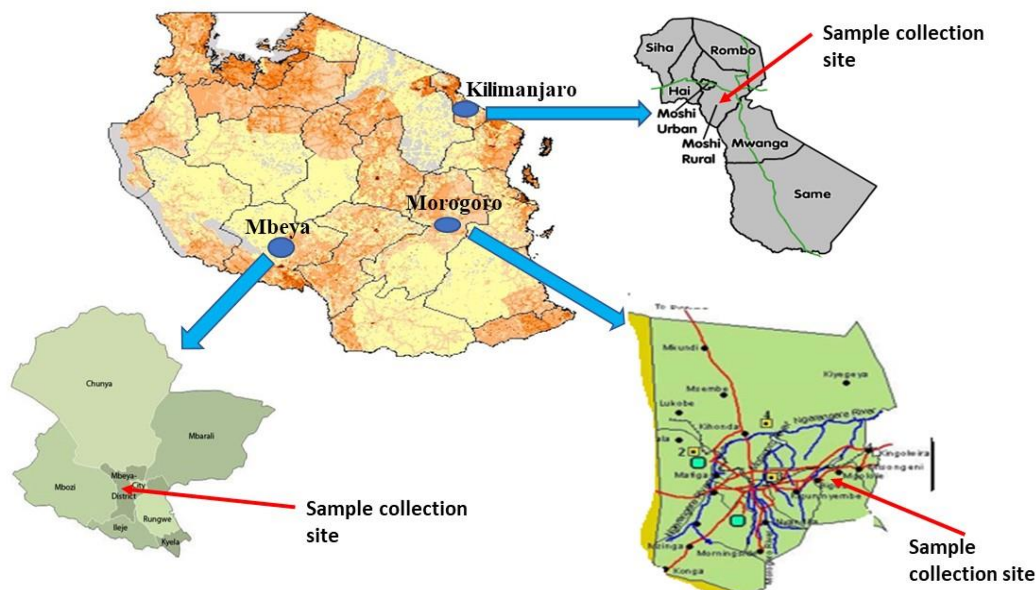

Figure 2: Map of Tanzania showing sample collection sites

3.2 Enrichment, growth and isolation of microbes

For each of the collected cereal-based beverage samples, four enrichment broths (each 20 mL) were prepared by using Nutrient Broth (Oxoid, UK) following the manufacturer's protocol. The 16 bottles containing the broth were sterilized and cooled in water bath at 45 °C for 10 to 15 min. The broths were inoculated with 1 mL of the stock sample such that each beverage sample had four enrichment solutions which were then incubated at 37 °C for 24 h. Then an aliquot of 0.5 µL of each of the enrichment culture was inoculated on plates with solid Nutrient agar; NA (Oxoid, UK) by the method of streaking and incubated at 37 °C. Each sample being studied had four replications and observations of the growing microbes on agar were done after 24 h, 48 h, 72 h and 96 h respectively. Growth on NA was intended to be a yard stick to gauge the presence or absence of microbes in the four cereal-based beverages. Simultaneously, slants were prepared in universal bottles using NA and individual isolates of the selected colonies were slanted and preserved at 4 °C for further analysis.

3.3 Characterization and identification of microbes

3.3.1 Macro-observations by sub-culturing of microbes

Agar plates containing Nutrient Agar (Oxoid, UK), Sabouraud Dextrose Agar (Oxoid, UK), De Man Rogosa and Sharpe (MRS) Agar (Biomérieux SA, France) and MacConkey Agar (Himedia Laboratories, India) were prepared according to the manufacturers' protocols as well

as routine lab techniques and inoculated with isolates from the slants. The plates were labelled and incubated at 37 °C for 24 - 48 h. The colonies were then physically observed (macro-morphology) using naked eyes for appearance/colour, edge and degree of growth (Desiye & Abegaz, 2013). Observations recorded were appearance (whitish or creamy) and edge (rough, smooth, round).

3.3.2 Micro-observations

Micro observation (micro-morphology) was achieved by employing the Gram Stain method. Smears of the isolates were prepared on microscope slides and stained following a routine laboratory Gram stain procedure. The slides were finally observed under a light microscope (Model CX21FS1, Olympus Corp. Tokyo, Japan) by using 100X objective lens. Micro-observations provided information on microbial shape (rod, ovoid, coccus), Gram Stain reaction (purple or red), microbial arrangement (single, pair, triple, tetrad, chains) and facilitated selection of working strains (isolates) through repeated streaking of a single colony on selective media to obtain pure isolates. These characteristics were recorded and used as criteria to make initial distinction among the isolates (purple colour - Gram positive microbes, red colour – Gram negative microbes, rod-shaped – likely lactobacilli and spherical shaped – likely yeasts) pending further tests to presume and affirm identity of isolates. Similarity of microbes led to random selection of representative isolates for further analysis.

3.3.3 Presumptive identification of isolates to genus level

Reasonable identification of microbial isolates to genus level was done based on key morphological and physiological characteristics (Kavitha *et al*., 2016; Kurtzman *et al*., 2011) after repeated streaking of isolates on selective agar for purification and microscopic examination. Such characteristics included appearance, shape, and growth in certain media (Desiye & Abegaz, 2013). Isolates were tentatively identified as *Lactobacilli* based on methods adopted by other researchers (Deshpande *et al*., 2017; Desiye & Abegaz, 2013; Kavitha *et al*., 2016) and use of Bergey's Manual of Systematic Bacteriology (Hammes & Hertel, 2009). Briefly, isolates were considered as LAB if they grew on MRS Agar, were Gram positive, rod-shaped or cocci, tested for growth at 15 °C and 45 °C, tested for growth in 4 and 6.5% NaCl and catalase negative. Isolates were tentatively identified as Yeasts based on morphological characterization as described by Kurtzman *et al*. (2011). Yeast colonies are mucoid, glittering, creamy colonies with entire edge. Isolates likely to be yeasts also grew on SDA as dome-like

colonies, were Gram positive, spherical or ovoid in shape with a fermenting malt smell. Tentative identification to genus level facilitated the grouping of the isolates into two groups; *lactobacilli* and yeasts for appropriate identification using the API® Systems (Biomérieux SA, Marcy - l'Etoile, 2016; Kurtzman *et al*., 2011).

3.3.4 Identification of isolates to genus and species level

Gram positive rods presumed to be *Lactobacillus spp*. were identified using the API® 50 CHL medium and the API® 50 CH strips according to the procedure described by the manufacturer (Biomérieux SA, Marcy - l'Etoile, France). Likewise, Gram positive, ovoid or spherical cells with the likelihood of being yeasts were identified with the aid of API® 20 C AUX which comprises the API® 20 C AUX strips and the API® C medium, as per the manufacturer's procedure.

3.4 Identification of isolates using molecular techniques

Extraction of genomic DNA from the sample isolates was done by employing thermal extraction method as described by Carriero *et al*. (2016). Adequate amount of pure isolate colonies was suspended in 200 µL of Nuclease free water (Fischer Scientific, Germany) in 1.5 mL Eppendorf tubes (Eppendorf, Hamburg Germany) and then placed in a water bath at 95 °C for 5 min and immediately transferred to ice for 5 min. This cycle was repeated three times before centrifugation at 13 000 x g for 10 min to precipitate all other debris except the DNA which remains as the supernatant. The DNA material was pipetted out into new tubes, spectrophotometrically quantified using Nano drop and preserved at -20 °C.

Amplification by polymerase chain reaction (PCR) of the 16S/23S rRNA spacer region which is a conserved region among *Lactobacilli* and other bacteria was done following the method described by Ségolène *et al*. (2002). In the case of yeast DNA extracts, PCR amplification of the ITS region which is the conserved region for fungi showing interspecies variability; was carried out according to the method described by Raja *et al*. (2017) with some modifications on the concentrations of the DNA template and the primers. The methodologies adopted for both lactobacilli and yeasts were designed to identify the microbes to the genus level. As such, it was important to sequence the PCR products.

Sequencing was carried out using ABI 3500 Genetic analyzer (Applied Biosystem ™, California, U.S.A) by Sanger method according to the manufacturer's instructions and protocol

(Inqaba Biotec Co. Ltd, South Africa). Sequence assembling and editing were performed in Bioedit software and the consensus sequences were blasted in the National Centre for Biotechnology Information (NCBI); https://blast.ncbi.nlm.nih.gov/Blast. All the sequences from this study and other selected reference sequences from the GenBank were aligned and phylogenetic analysis were performed in the Molecular Evolutionary Genetics Analysis software (MEGA X).

3.5 Laboratory preparation of probiotic beverages

3.5.1 Screening of the LAB strains for selection of probiotic starter cultures

Three pure LAB strains identical to the ones isolated as main LAB from the four-local cereal-based beverages were tested for viability under controlled fermentations at 37 °C for 24 h. This was done purposely to determine suitable probiotic LAB strains for use as probiotic starter culture for each beverage. Specifically, *Lactobacillus brevis, Pediococcus pentosaceus* and *Lactobacillus plantarum* obtained from Belgian Co-ordinated Collections of Microorganisms-Laboratory of Microbiology Ghent (BCCM-LMG), were tested for development of *Kindi, Kimpumu, Mbege and Togwa*.

3.5.2 Preparation of the probiotic cereal-based beverages

The laboratory preparation of either probiotic *Kindi*, *Kimpumu*, *Togwa* or *Mbege* was done by mimicking the traditional preparation methods with some modifications in certain steps; based on procedure adopted by Di Stefano *et al*. (2017). Raw materials namely; maize and finger millet (*Kindi*), finger millet (*Kimpumu*), maize and finger millet (*Togwa*) and bananas and finger millet (Mbege) were used. Based on the prior screening results, selected probiotic starter cultures were *L. brevis* (*Togwa*), *P. pentosaceus* (*Kindi*), and *L. plantarum* (*Kimpumu and Mbege*), respectively. All fermentations were conducted in the laboratory in Erlenmeyer flasks at 37 °C for 24 h under sterile conditions.

(i) Preparation of Kindi

The ingredients used for production of the beverage are maize flour, finger millet malt flour and water. *Kindi* was prepared in the ratio of 1:10:0.5 (maize flour: water: millet malt flour). Maize (*Zea mays*) grains after being washed and dried were ground to get maize flour which was soaked in cold water for an hour. The slurry was cooked in hot water at a temperature of

70 – 80 °C for 15 min while stirring it with a wooden handle and then cooled to between 45 - 50 °C. Finger millet malt flour was then added, and the mixture was stirred for 20 min until the temperature was lowered to 37- 40 °C. The mixture was inoculated and left to ferment at 37 °C for 24 h to get fresh *Kindi*. Figure 3 shows the flow diagram for probiotic *Kindi* production.

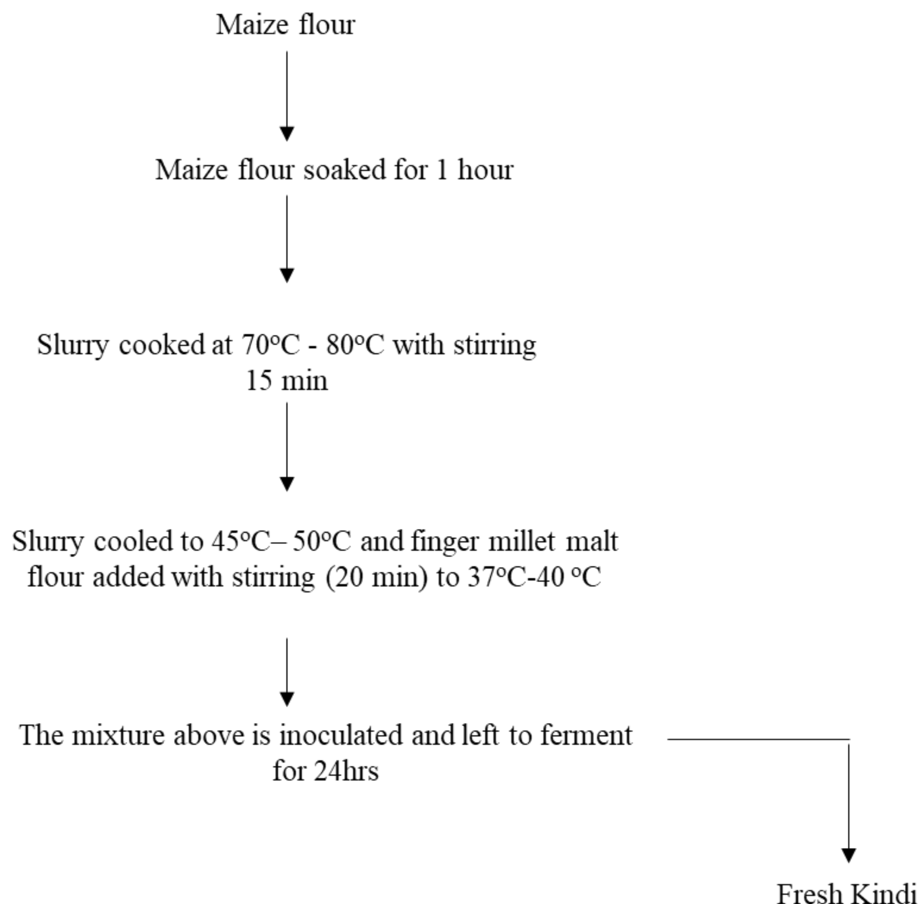

Figure 3: Flow diagram for preparation of probiotic *Kindi*

(ii) Preparation of Kimpumu

The production of *Kimpumu* involves the use of finger millet malt flour and water as ingredients. *Kimpumu* was prepared in the ratio of 1:10 (millet malt flour: water). The process starts with soaking of the millet grains in cold water for one day. The soaked millet grains were allowed to drain off water while germinating for two days. The millet malt was then dried on the sun for one day followed by winnowing and milling to malt flour. Millet malt flour was then cooked for 15 min in water at 60 - 80 °C and the resulting gruel cooled to 37 - 40 °C in 10-20 min. The cooled gruel was inoculated with the probiotic bacteria and left to ferment at 37 °C in 24 h when fresh *Kimpumu* was obtained. Figure 4 indicates the flow diagram for probiotic *Kimpumu* production.

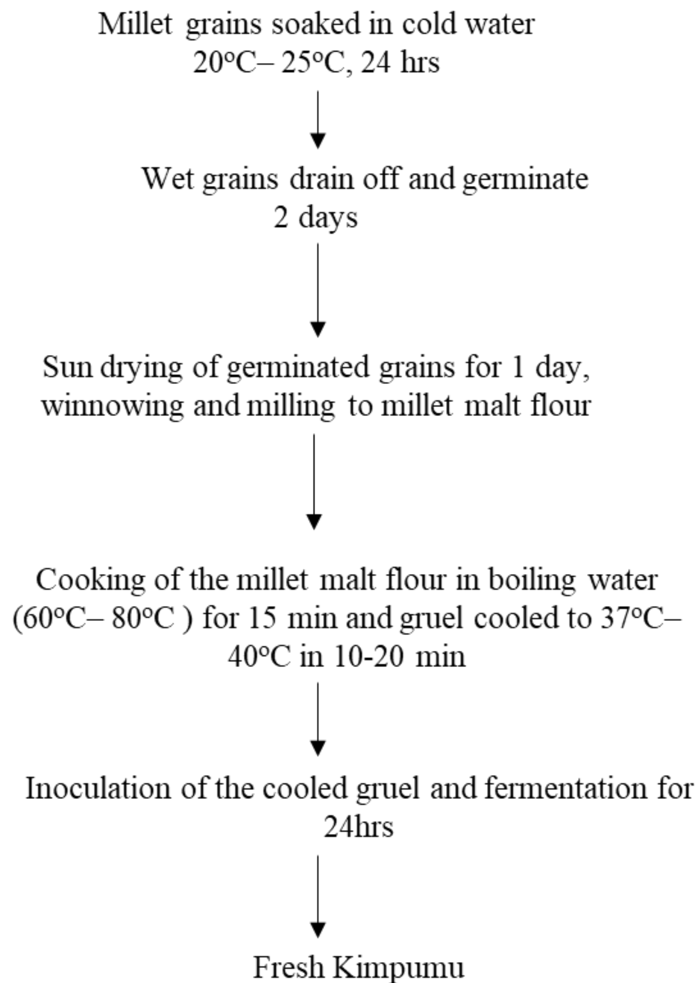

Figure 4: Flow diagram for probiotic *Kimpumu* processing

(iii) Preparation of Togwa

Preparation of *Togwa* involved the use of three ingredients namely finger millet malt flour, maize flour, and water. The beverage ingredients were mixed in the ratio of 1:10 (maize flour: water and millet malt flour: water). The production process started with winnowing of the finger millet grains and soaking them in cold water for one day. The soaked millet was allowed to germinate while draining off water for 3 - 4 days. The wet (moist) germinated grains were sun dried for one day and then milled to get millet malt flour. The millet malt flour was cooked for 15 min in hot water at 50 - 70 °C while being stirred and then mixed with pre-cooked maize gruel to uniform consistency and final temperature of 37 - 40 °C. Fresh *Togwa* was obtained after inoculation of the mixture and fermentation at 37 °C for 24 h. Figure 5 illustrates the flow diagram for probiotic *Togwa* production.

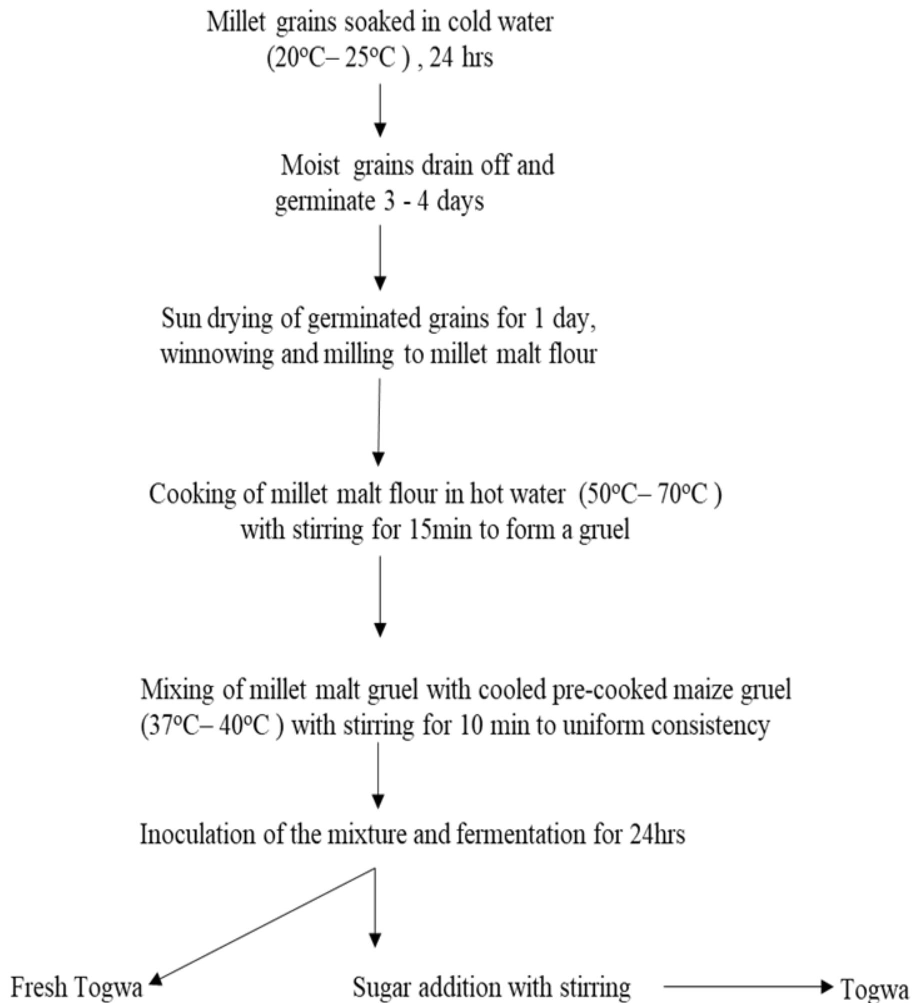

Figure 5: Probiotic *Togwa* production flow diagram

(iv) Preparation of Mbege

The main ingredients; ripe bananas, millet malt flour and water (excluding *Msesewe* and *Nganana*) were mixed in the ratio of 1:1 (ripe bananas: water) and 1:10 (millet malt flour: water). *Mbege* was prepared by first cooking ripe bananas in hot water (50 - 70 °C) in a metal vessel for 15-30 min and cooling the cooked bananas to ambient temperature in 20-30 min. Cold water was added to the cooled bananas and stirred for 5-10 min. The cooked banana slurry was then filtered off and the resulting filtrate was mixed with cooked millet malt flour (in gruel form) while being stirred at 70 - 80 °C and the mixture cooled to 37- 40 °C. The mixture was finally inoculated with a probiotic bacterium and fermented at 37 °C for 24 h to obtain fresh *Mbege*. Figure 6 shows the flow diagram for probiotic *Mbege* production.

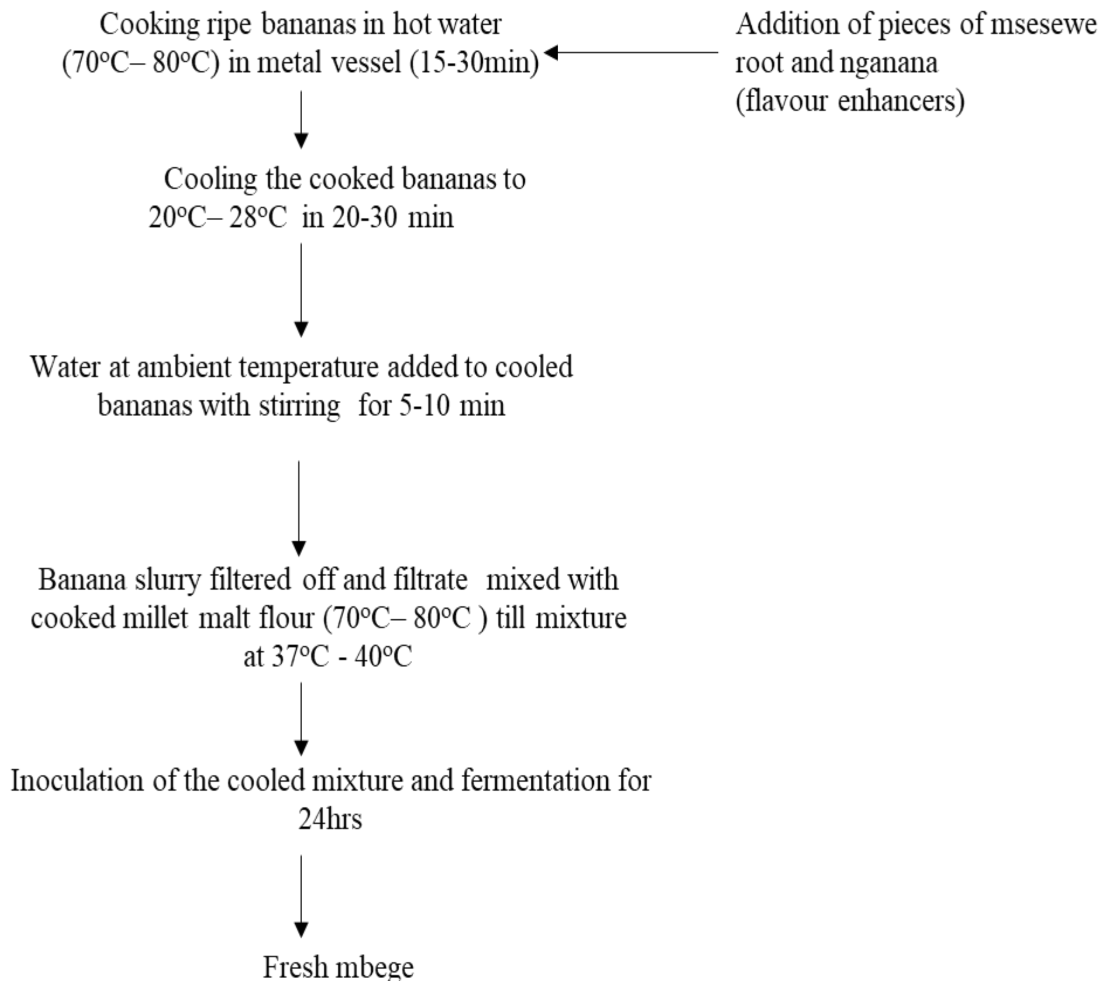

Figure 6: Probiotic fresh *Mbege* production flow diagram

3.6 Assessment of the probiotic potential of the cereal-based beverages produced under controlled fermentations

3.6.1 Preparation of inocula for cereal beverage fermentations

Three pure cultures of LAB strains (*L. brevis*, *P. pentosaceus*, and *L. plantarum*) stored at -20 °C in 15% glycerol were activated by allowing them to equilibrate with ambient temperature (25 °C). Then 100 µL of each pure culture were respectively transferred to 10 mL of MRS broth and incubated at 37 °C for 48 h. Again, 100 µL of each LAB strain were pipetted out into 10 mL of new MRS broth in a screwed universal bottle and preserved at 4 °C as Stock culture (kept for one month). Working culture for each LAB strain was taken from the stock culture and incubated at 37 °C for 24 – 48 h and then initially centrifuged at 5000 x g for 10 min at 4 °C to remove the MRS medium and subsequently twice washed with sterile normal saline water by centrifuging at 6000 x g for 5 min at 20 °C, then decanting the supernatant and finally the

pellet was re-suspended in new sterile normal saline water ready for use. The probiotic bacteria were then inoculated in the cereal substrates with a population of 7 – 8 log cfu/mL (Charalampopoulos *et al*., 2002; Freire *et al*., 2017).

3.6.2 Enumeration of lactic acid bacteria populations and pH measurements during fermentation

This was performed following the methods of Freire *et al*. (2017) and Charalampopoulos *et al*. (2002) with some modifications. Briefly, LAB populations were determined by taking 1 mL samples from each fermentation flask at 0, 6, 18, 24, 30 and 48 h of fermentation; and then 10-fold diluted in sterile normal saline water and plating them in duplicates on MRS agar employing a pre-calibrated pipette followed by anaerobic incubation at 37 °C for 48 h in anaerobic jars (BBL, Oxoid). Pure culture of the probiotic bacteria of interest (either *X, Y,* or *Z*) was inoculated on the same agar medium to act as positive control while distilled water inoculated on identical agar medium acted as a negative control. Colony enumeration was performed to ascertain whether or not the products attain or surpass the required therapeutic minimum dose of 10^6 cfu/mL viable cells in the products, and were expressed as cfu/mL. The pH of each sample at the above given time intervals was measured using a pH-meter (pH 510, Wagtech International, UK).

3.7 Sensory evaluation

Sensory evaluation of fermented probiotic cereal-based beverages was performed by means of consumer acceptance test as described by Freire *et al*. (2017). Fifty untrained panelists were selected based on their drinking habit of the four local fermented beverages (*Kindi, Kimpumu, Togwa* and *Mbege*), aged between 15 and 60 years, and involved staff and students at SUA and local drinkers at the research sites. Local drinkers comprised 48% and non-local drinkers 52% while 76% were males and 24% females. Each panelist received the four different coded samples. Randomized 15 mL samples were handed out in clear 50 mL glasses at between 4 and 25 °C. The consumers rinsed their mouths with portable water between tastings. With the help of test score sheets (Appendix 1); samples were evaluated for appearance, taste, color, texture, aroma and general acceptability based on 7-point hedonic scale scores ranging from like extremely (1) to dislike extremely (7).

3.8 Shelf-life studies

3.8.1 Shelf life estimation

The storage time of the fermented cereal-based beverage was defined as the length of time at 4 °C during which pH remained above 4.0 and the viable cell counts was above 10^6 cfu/mL (Angelov *et al*., 2006; Hassan *et al*., 2012).

The shelf-life at ambient temperature was defined as the length of time at 25 °C during which pH remained above 3.5 and the viable cell counts was above 10^6 cfu/mL (Salmerón *et al*., 2015). The pH value of 3.5 was chosen as the minimum at ambient temperature because it is the threshold value observed in most cereal-based fermented beverages (Mukisa *et al*., 2017; Salmerón *et al*., 2015).

Refrigerated storage was done for 28 days and ambient temperature storage for 5 days with observations of pH and the viability of the starter cultures every 7 days (4 °C) and 24 h (25 °C). The products were packaged in food grade polypropylene (PP) screw caped bottles.

3.8.2 Lactic acid bacteria counts and pH measurements at refrigerated storage

This was done according to Di Stefano *et al*. (2017) with some modifications adapted from Charalampopoulos *et al*. (2002) and Freire *et al*. (2017). LAB counts were determined by taking 1 mL samples from each storage flask at 0, 7, 14, 21, and 28 days; and then 10-fold diluted in sterile normal saline water and plating them on MRS agar followed by incubation at 37 °C for 48 h. The CFU were then counted and results were expressed as cfu/mL. The pH was measured using a pH-meter (pH 510, Wagtech International, UK).

3.8.3 Lactic acid bacteria counts, and pH measurements at ambient temperature

The same method as in Section 3.8.1 above was followed for viable cell counts and pH measurement. However, 1 mL of each sample was withdrawn at 0, 24, 48, 72, 96, and 120 h of storage.

3.9 Microbiological analysis for presence/absence of pathogens

Microbiological analysis for safety of the probiotic cereal-based beverages i.e., detection of indicator pathogens e.g., *Salmonella, Shigella* and *Escherichia coli* was done using selective media of the pathogens. Selective media used were BGA (CM0263) for *Salmonellae*,

Salmonella-Shigella agar-SSA (CM0099) for *Shigella spp*., and VRBGA (CM0485) for *E. coli*. One milliliter was drawn from each of the probiotic samples at regular intervals during shelf-life studies at 25 °C and 4 °C, respectively. The drawn sample was 10-fold diluted and inoculated on selective media (prepared following the manufacturer's protocol-Oxoid, UK) specific for the pathogens and incubated at 35 °C for 24 h (32 °C for 24 - 48 h for *E. coli*). Pure cultures of the microbes of interest were inoculated on selective agars as positive controls while distilled water inoculated on the agars was taken as negative controls. Samples were taken after every 24 h during storage at 25 °C and at the end of each week during storage at 4 °C. The safety of the probiotic beverages was indicated by the non-growth of the pathogens on the respective media.

Tests for spoilage microbes such as yeasts and molds were not performed during storage for shelf-life studies because the beverage containers were vacuumized at the head space so that yeasts and molds which are aerobes couldn't grow. The only concern was safety of the beverages as *Salmonella, Shigella* and *E. coli* are anaerobes and could grow under such environment.

3.10 Data analysis

All data on fermentations of cereal-based beverages, and shelf-life studies at 25 and 4 °C were subjected to Analysis of Variance (ANOVA) by employing GenStat 15th edition statistical software (VSN International) in order to ascertain the means of the variables (pH and viable cell counts) at various fermentation and storage times and the means of the variables based on beverage type. Variable means were differentiated by employing the Student-Newman-Keuls test (SNKT) at 5% significance level of probability.

Data collected on sensorial evaluations (acceptance tests) were also analyzed by ANOVA making use of GenStat 15th edition statistical software (VSN International) to determine the means of the cereal-based beverage quality attributes and the means separation was done by using the Student-Newman-Keuls test (SNKT) at 5% significance level of probability.

Data collected on sensorial evaluation by making use of the Just-about-right measure were analyzed by MS-Excel 2019 analytical package.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Acidity and pH of the cereal-based products

The pH of the Kindi, Kimpumu, Togwa and Mbege were 3.6, 4.0, 3.6 and 4.0 respectively; indicating low acidity in all beverages. The low acidity or pH of all the fermented cereal-based beverages implies that the production of such beverages results to products and by products with low pH values. Most microbes grow well at optimal pH values or neutral pH values; 6.5-7.0 (James *et al*., 2005) than those observed in the sampled beverages. It has been empirically shown that some microbes such as LAB can grow and survive at pH values from 5 and below (Waters *et al*., 2015). LAB activities during the fermentation of carbohydrates also produces lactic acid as the main end product, thus decreasing the pH of the resulting product (Achi & Asamudo, 2019; Kohajdová & Karovičová, 2007). On the other hand, literature indicates that yeasts grow well in a wide range of acidic pH (Kurtzman *et al*., 2011; Mukisa *et al*., 2017; Todorov & Holzapfel, 2014). LAB and yeasts have also been observed to be the major groups of microorganism which participate in the fermentation of most of the cereal-based foods and beverages (Achi & Asamudo, 2019; Aka *et al*., 2014). The presence of low pH in the end products due to production of lactic acid and other organic acids is ideal for their growth and exponential increase. Consequently, the observation of low pH values in the tested local cereal-based beverages indicate the possibility of the predominant growth and proliferation of acidophilic microbes such as LAB and yeasts during the spontaneous fermentations.

4.2 Isolation of microbiota

The cloudiness in the enrichment broths indicated increase in microbial cells that was reflected in high degree of colonial growth on the plates. The growth of the same types of colonies persisted on the plates. The degree of growth of the same types of colonies increased with incubation time. The existence of similar colonies for all beverage samples showed the possibility of having the same types of microflora participating in the fermentation of the respective beverages. Isolation resulted to 64 unknown isolates that were slanted.

4.3 Macro observations

Smooth round colonies with whitish or creamy colors were observed on NA, MRS and SDA but colonies growing on MAC appeared smooth and colorless or transparent other than having the red or pinkish colour characteristic for lactose fermenters (Hardy Diagnostics, 2020; Tankeshwar, 2019). As such lactose fermenters particularly Enterobacteriaceae such as *E. coli*, *Klebsiella* or *Enterobacter spp*. were absent in the test samples (Hardy Diagnostics, 2020; Tankeshwar, 2019). There was possibility of growth of *Salmonella spp*. and *Shigella spp*. which are non-lactose fermenters in *Togwa* and *Mbege* following the growth of colourless and transparent colonies (Hardy Diagnostics, 2020; Tankeshwar, 2019). Consequently, it was possible to differentiate the colonies based on appearance/colour and shape or edge. Results on appearance of colonies are given in Tables 5 and 6. Plate 2 shows growth of LAB colonies and yeast colonies on MRS and SDA respectively.

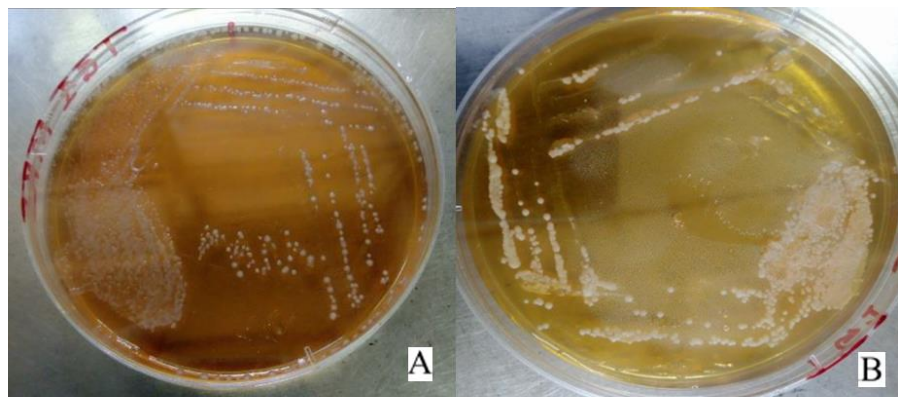

Plate 2: Smooth round colonies of *lactobacilli* on De Man, Rogosa and Sharpe Agar (A), dome-like colonies of yeasts on Sabourad Dextrose Agar (B)

4.4 Micro-observations

Togwa and *Mbege* contained both gram-positive and gram-negative microbes. However, gram negative microbes were not found in *Kindi* and *Kimpumu*, respectively. Gram positive rods (singles, pairs and triples) grew predominantly in NA and MRS for all sample isolates whereas gram positive, ovoid cells (singles, pairs, triples, clusters) grew mostly in SDA for all isolates. Gram negative coccoid rods, which are colorless and transparent grew predominantly in MacConkey agar for *Togwa* and *Mbege* isolates only. Among the 64 isolates microscopically observed; 12 were gram negative coccoids and 52 were gram positive rods and ovoid cells. Plate 3 presents the appearance of LAB and yeasts under the microscope.

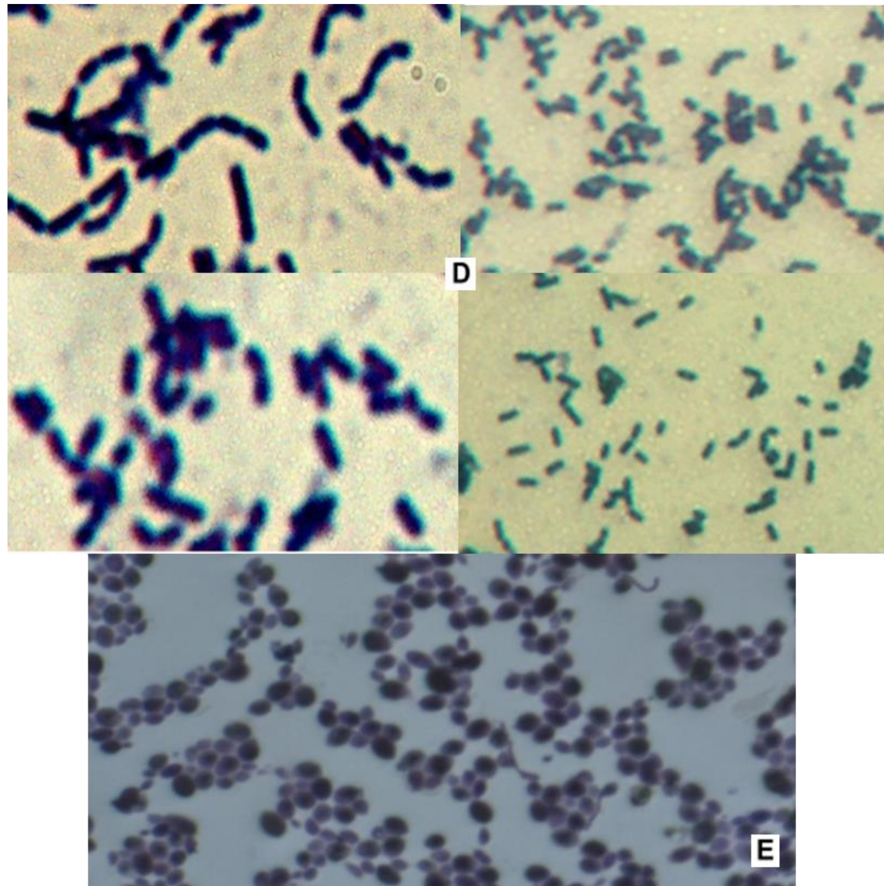

Plate 3: Appearance of LAB (D) and Yeasts (E) under the Microscope (100X magnification)

These results show that Gram-positive rod-shaped bacteria in pairs, triples or singly occur in all beverage samples. These are likely to be various species of *lactobacilli* which are important in carrying out the acidic fermentations of the beverages (Abegaz, 2007; Oyedeji *et al*., 2013). Ovoid or spherical gram-positive cells in clusters, pairs or singly with a fermenting malt smell were also observed in all beverage samples showing the possibility of the participation of fungi/yeasts in the fermentations of these beverages (Mugula *et al*., 2003a; Mukisa *et al*., 2017). The presence of gram-negative coccoid rods in Togwa and Mbege isolates show the possible existence of pathogenic or spoilage microbes in the beverages (Abegaz, 2007; Mugula *et al*., 2003a). However, these are normally eliminated by the decreased pH of the substrate and production of other antimicrobial metabolites as well as the byproducts of the fermentation process (Aka *et al*., 2014; Devi & Halami, 2017). Tables 5 and 6 summarize the important morphological, physiological and biochemical results obtained for representative isolates.

Table 5: Morphological, physiological and biochemical characterization of LAB isolates

	LAB Isolates and results										
Characteristics	**T1a (3)**	**T2b**	**T3d**	**KM2a (3)**	**KM4b (1)**	**KD1a (2)**	**KD2b**	**KD4c (2)**	**MB1a (2)**	**MB2b**	**MB4d (2)**
Morphological											
Colour/appearance	Whitish	Whitish	Whitish	Whitish	Whitish	Whitish	Whitish	Whitish	Whitish	Whitish	Whitish
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram reaction	+	+	+	+	+	+	+	+	+	+	+
Physiological											
Growth at different temperatures											
15ºC	+	+	+	+	+	+	+	+	+	+	+
45ºC	-	-	-	-	-	+	+	+	-	-	-
Growth in NaCl											
4%	-	-	-	-	-	+	+	+	-	-	-
6.5%	-	-	-	-	-	+	+	+	-	-	-
Catalase test	-	-	-	-	-	-	-	-	-	-	-
Biochemical (49 sugars involved only important shown)											
N-Acetyl Glucosamine	+	+	+	+	+	+	+	+	+	+	+
Amygdalin	+	+	+	+	+	+	+	+	+	+	+
Arbutin	+	+	+	+	+	+	+	+	+	+	+
Esculine ferric citrate	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+	+	+	+
D-Cellobiose	+	+	+	+	+	+	+	+	+	+	+
D-Maltose	+	+	+	+	+	+	+	+	+	+	+
D-Lactose	+	+	+	+	+	+	+	+	+	+	+
D-Melibiose	+	+	+	+	+	+	+	+	+	+	+
D-Saccharose	+	+	+	+	+	+	+	+	+	+	+
D-Trehalose	+	+	+	+	+	+	+	+	+	+	+

Note: + = positive, - = negative. T1a, T2b…MB2b, MB4d = codes for *Lactobacillus* isolates. T1a (3) = *L. pentosus*, T2b = *L. plantarum*, T3d = *L. brevis*, KM2a (3) = *L. plantarum*, KM4b (1) = *L. plantarum*, KD1a (2) = *P. pentosaceus*, KD2b = *P. pentosaceus*, KD4c (2) = *P. pentosaceus*, MB1a (2) = *L. plantarum*, MB2b = *L. plantarum*, MB4d (2) = *L. plantarum.* T3c = *Lactococcus lactis ssp lactis* (not shown). Bracketed number like (3) shows number of other isolates with same identification.

Table 6: Morphological and biochemical characterisation of yeast isolates

	Yeast Isolates and results											
Characteristics	**YIA (2)**	**YIB (2)**	**YIC**	**YID**	**YIE (1)**	**YIF**	**YIG**	**YIH (2)**	**YII (1)**	**YIJ (2)**	**YIK (1)**	**YIL (2)**
Morphological												
Colour/appearance	Creamy	Creamy	Creamy	Creamy	Creamy	Creamy	Creamy	Creamy	Creamy	Creamy	Creamy	Creamy
Shape	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid
Gram reaction	+	+	+	+	+	+	+	+	+	+	+	+
Biochemical (19 sugars involved only important shown)												
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	-	-	-	+	+	+	-	+	+	+	+	+
D-Adonitol	+	+	+	-	-	-	-	+	-	+	-	+
Xylitol	-	-	-	-	-	-	-	+	-	+	-	+
D-Galactose	+	+	+	+	-	+	+	+	-	+	+	+
D-Maltose	+	+	+	+	-	+	+	+	-	+	+	+
D-Saccharose	-	-	-	+	-	+	+	-	+	+	+	+
D-Trehalose	+	+	+	+	+	+	+	+	+	+	-	+
D-Melezitose	+	+	+	+	-	-	-	-	-	-	-	-
D-Raffinose	-	-	-	+	-	-	-	-	-	-	-	-

Note: + = positive, - = negative. YIA, YIB, …YIK, YIL = codes for yeast isolates. YIA (2) = *C. tropicalis*, YIB (2) = *C. tropicalis*, YIC = *C. tropicalis*, YID = *C. tropicalis*, YIE (1) = *Candida zeylanoides*, YIF = *Candida albicans*, YIG = *Cryptococcus gattii*, YIH (2) = *C. tropicalis*, YII (1) = *Rhodotorula minuta*, YIJ (2) = *Candida ciferrii,* YIK (1) = *Candida dubliniensis,* YIL (2) = *Candida ciferrii.* Numbers in brackets like (2) shows number of other isolates with same identification.

4.5 Microbial isolates identification to genus and species level

4.5.1 Identification of lactic acid bacteria

Identification results of the generated biochemical profiles of the isolates indicate that Togwa isolates were predominantly *Lactobacillus spp*. namely *L. pentosus, L. brevis, L. plantarum,* and *Lactococcus lactis ssp lactis*. In the case of Kindi isolates; the dominant LAB was *Pediococcus pentosaceus.* The main LAB in Kimpumu and Mbege isolates was *L. plantarum.* Illustration 1 shows the biochemical changes in the API 50 CH strips which led to generation of biochemical profiles for identification of isolates as summarized in Table 7.

Illustration 1: Appearance of the API 50 CH strips inoculated with an unknown *Lactobacillus* strain before (A) and after incubation (B)

Table 7: *Lactobacillus* species isolated from the cereal-based beverages

Beverages	Fermenting Species
Togwa	*Lactobacillus pentosus,* *Lactobacillus brevis* *Lactobacillus plantarum* *Lactococcus lactis ssp lactis*
Kindi	*Pediococcus pentosaceus*
Mbege	*Lactobacillus plantarum*
Kimpumu	*Lactobacillus plantarum*

The major implication of the identified *Lactobacillus spp*. from the collected samples of local cereal-based beverages is that spontaneous fermentation is carried out by species of the three genera of LAB namely *Lactobacillus, Lactococcus* and *Pediococcus*. However, the most dominant LAB in Togwa were *L. pentosus, L. brevis,* and *L. plantarum.* In other beverages; Kindi, Kimpumu and Mbege, the predominant LAB were *P. pentosaceus* and *L. plantarum,* respectively. Mukisa *et al*., (2017) isolated some LAB namely *L. plantarum, Weissella confusa, Lc. lactis and L. fermentum* from Obushera; a traditional sorghum malt fermented beverage in Uganda. Other studies on fermented cereal-based beverages like that of Ogi from fermented maize has shown the presence of *L. plantarum* and *Lc. lactis* among others in the end product (Oyedeji *et al*., 2013). Abegaz (2007) was able to isolate LAB of the genera *Lactobacillus*, *Pediococcus* and *Weissella* as dominant LAB from the fermentation of Borde; an Ethiopian spontaneously fermented low or non-alcoholic cereal-based beverage.

Lactic acid bacteria genera of *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus* and *Weissella* have also been found to dominate the fermentations of two Zambian beverages known as Chibwantu and Munkoyo which are made from maize and sometimes with addition of millet or sorghum (Schoustra *et al*., 2013). Mugula *et al*. (2003a) reported on the presence of *L. fermentum, L. plantarum, L. brevis, L. cellobiosus, P. pentosaceus* and *W. confusa* as the fermenting LAB in spontaneously fermented Togwa. Generally, LAB genera taking part in the fermentations of the four-local cereal-based beverages were identical to those of the previous studies narrated above though in the earlier studies there were some additional genera such as *Weissella, Leuconostoc* and *Streptococcus*. The slight difference in the participating genera is attributed to substrate composition, microbial structure differences and surrounding environmental conditions in the sites of beverage preparation, preparation methods of the beverages, metabolites produced during fermentation, the pH of the end products and the initial microbial load in the substrates (Schoustra *et al*., 2013). It is also worth noting that among the LAB participating in the fermentations of the four local cereal beverages; *L. plantarum, L. brevis, L. pentosus,* and *P. pentosaceus* have been proven to be probiotics (Bourdichon *et al*., 2012).

The LAB isolated from the four cereal-based beverages are important for carrying out the lactic fermentations of the products which leads to production of lactic acid as the main end product (Aka *et al*., 2014; Kalui *et al*., 2010; Mokoena *et al*., 2016). The lactic acid and other organic acids produced such as acetic acid, butanoic acid, propionic acid invariably increase the acidity

and lowers the pH of the products to below pH 4.0 creating inhibitive conditions for the growth of other microbes especially pathogenic and spoilage microbes (Aka *et al*., 2014; Oyedeji *et al*., 2013). In this way, they render the fermented cereal-based products safe for human consumption (Kumari *et al*., 2015; Mokoena *et al*., 2016). Besides, the LAB activities during the fermentation of cereal-based beverages produces volatile flavour compounds such as acetaldehydes, acetates, acetone and acetoin; which improves the odour and tastes of the fermented cereal beverages (Mukisa *et al*., 2017). The LAB as well improves the textural properties of the beverages (Marsh *et al*., 2014; Peyer *et al*., 2016). As a result, consumers of the fermented cereal beverages; though naturally fermented, derive health benefits from some probiotics present in the products such as those mentioned previously (Kalui *et al*., 2010). The healthy benefits include prevention and cure of gastro-intestinal disorders, lactose intolerance, high blood pressure (BP), diabetes, coronary heart disease, cancer and obesity (Kechagia *et al*., 2013; Marco *et al*., 2017).

4.5.2 Identification of yeasts

The seven-digit numerical profiles of yeast isolates were key in identification of yeast species or strains. The results indicate that Togwa isolates were all identified as *C. tropicalis*; Kindi isolates were *Cryptococcus gattii*, *C. tropicalis* and *Rhodotorula minuta*. On the other hand, *C. zeylanoides*, *C. albicans*, and *C. tropicalis* were identified in Kimpumu while *Candida ciferrii* and *Candida dubliniensis* were identified in Mbege. Illustration 2 depicts the change to turbidity for some cupules of the API 20 C AUX strips, which led to generation of the 7-digit numerical profiles of yeast isolates and Table 8 depicts the results explained above.

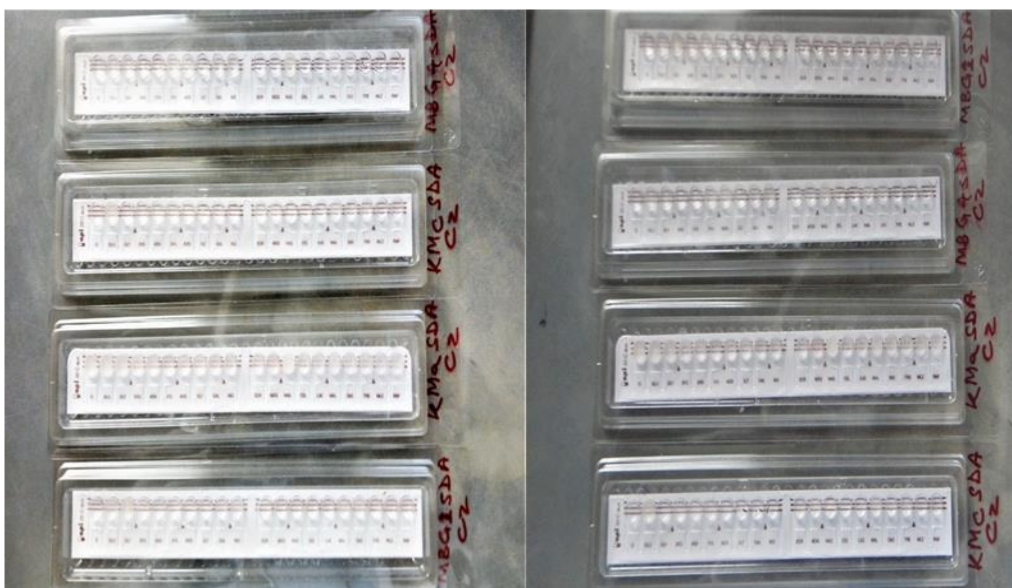

Illustration 2: Clear cupules (left) and some turbid cupules (right) after incubation

Table 8: Yeast species isolated from the cereal-based beverages

Beverage	Fermenting Species
Togwa	*Candida tropicalis*
Kindi	*Cryptococcus gattii*
	Candida tropicalis
	Rhodotorula minuta
Mbege	*Candida ciferrii*
	Candida dubliniensis
Kimpumu	*Candida zeylanoides*
	Candida tropicalis
	Candida albicans

The results in Table 8 elucidate the empirical fact that during fermentation of the four cereal-based beverages, *Candida* was the major genus of yeasts participating in such fermentations. The most predominant yeast in *Togwa* was *C. tropicalis*. On the other hand, *Rhodotorula minuta, Cryptococcus gattii*, and *C. tropicalis* were isolated in *Kindi.* The most proliferating yeasts in *Kimpumu* were *C. zeylanoides, C. tropicalis* and *C. albicans* whereas in *Mbege*, *C. ciferrii* and *C. dubliniensis* were predominant. According to Johansen *et al*. (2019), a large number of yeast *spp*. are present in the early stages of the spontaneous fermentations. This also presents large diversity at species level. Similarly, *C. tropicalis* has been reported to dominate in the fermentation of *Togwa* in Tanzania (Mugula *et al*., 2003a) and Fura; a pearl millet-based food (Pedersen *et al*., 2012) in West Africa, respectively. Likewise, dominance of the *Candida spp*. was observed in three spontaneously fermented Nigerian cereal-based beverages of *Burukutu* (sorghum & cassava), *Kunu-zaki* (maize & red sorghum) and *Ogi* (maize & sorghum/millet) with almost 40% of the isolates identified as *Candida spp*. (Ogunremi *et al*., 2015). In another study, *Candida spp.* were among the predominant yeast species taking part in the fermentations of many traditional cereal-based products (Mukisa *et al*., 2017). This further support findings from this study where *Candida spp*. were isolated from the cereal-based beverages. Although yeasts of the genus *Candida* have been associated with some diseases (candidiasis) in human and animals; recent development in research for beneficial microbes have indicated that some *Candida spp*. (family Saccharomycetaceae) participate in various food fermentations and have been included in the microbial food culture list for food fermentations (Bourdichon *et al*., 2012). The slight difference in some of the *Candida spp*. observed lies in the specificity of the microbial strains to the cereal substrates and other factors as previously elaborated in the case of LAB above. *Candida albicans* is an opportunistic pathogen though a non-harmful member of the microflora in heathy individuals. Some studies have also shown that various yeast *spp*. (non-*Saccharomyces spp*.) such as those of the genera

of *Candida, Debaryomyces, Pichia* and *Rhodotorula* have probiotic potential because of their ability to withstand the harsh conditions and colonize the gastro-intestinal tract during assays of various mammalian cell models (Lara-Hidalgo *et al*., 2017; Pedersen *et al*., 2012). The empirical fact that fermentations of cereals involving LAB and yeasts results to decreased pH in the range 3.5-4.0 favours the proliferation of such microbes while others including pathogenic ones are inhibited in such low acidity (Aka *et al*., 2014; Oyedeji *et al*., 2013). In cereal-based beverages, yeasts may originate from the raw materials (cereals, water), beverage handlers, processing equipment and surrounding environment (Johansen *et al*., 2019; Lara-Hidalgo *et al*., 2017). Yeasts are important in spontaneous cereal fermentations because they provide amino acids, peptides, and vitamin B_{6}, which are important for the growth of LAB that ferment the cereals. They are also producers of flavor compounds such as acetaldehydes, alcohols, esters, organic acids and acetates (Johansen *et al*., 2019; Lara-Hidalgo *et al*., 2017). Yeasts have shown resistance to gastric conditions by surviving to pH as low as 1.5 - 2.5 and showing good viability in the presence of bile salts (Johansen *et al*., 2019; Lara-Hidalgo *et al*., 2017). In cereal-based beverages; they produce phytase enzyme that is important in the GIT for breakdown and release of phosphorus and other divalent ions such as Fe, Zn, Mn, Ca and Mg (Lara-Hidalgo *et al*., 2017; Moslehi-Jenabian *et al*., 2010). Some yeast cells such as *Pichia kudriavzevii* have probiotic potential in cereal beverages and have been found to inhibit cholesterol absorption in the human and thus prevent risks of coronary heart disease (Lara-Hidalgo *et al*., 2017; Rai *et al*., 2019). Presence of yeasts in spontaneous fermentation of cereal-based products provides possibility for antagonistic yeasts to be used to inhibit the growth of pathogenic bacteria (Lara-Hidalgo CE *et al*., 2017; Rai *et al*., 2019). Co-existence of yeasts and LAB is a characteristic of the spontaneously fermented non/low alcoholic cereal-based beverages. Likewise, several interactions between several groups of microorganisms, i.e., yeast-yeast, bacteria-bacteria, yeast-bacteria and yeast-mold occur (Johansen *et al*., 2019). Moreover, *S. cerevisiae* and *S. cerevisiae subsp. boulardii* are the only known probiotic yeasts to date (Lara-Hidalgo *et al*., 2017; Moslehi-Jenabian *et al*., 2010; Ogunremi *et al*., 2015; Pedersen *et al*., 2012). Therefore, further research on isolation and characterization of yeasts from spontaneously fermented cereal-based beverages is mandatory to uncover novel species from various fermented cereal-based beverages.

The novelty of the results above is on isolation and identification of fermenting microflora specifically in the *Kindi*, *Kimpumu* and *Mbege*. This is the first study involving isolation and characterization of probiotic microbes from such beverages. *Lactobacillus pentosus* is a novel

LAB isolated from Togwa. Novel yeasts isolated from *Kindi* and *Kimpumu* are C. *gattii* and *R. minuta,* as well as *C. zeylanoides* and *C. albicans*, respectively. Furthermore, *C. ciferrii* and *C. dubliniensis* are novel yeasts isolated from *Mbege*. It has been proven that *L. pentosus* isolated in this study has been characterized to be probiotic (Bourdichon *et al*., 2012). Moreover, *R. minuta* has been found to have probiotic potential (Johansen *et al*., 2019; Lara-Hidalgo *et al*., 2017; Pedersen *et al*., 2012). Most of the *Candida spp*. are innocuous, but *C. albicans* have been found to be the potent agent of Candidiasis causing almost 47% of total yeast infections globally (Johansen *et al*., 2019). However, *C. albicans* is found in a few indigenous African fermented foods and beverages as a contaminant from food handlers during processing of the cereal-based beverages. Other *Candida spp*. such as *C. zeylanoides, C. ciferrii, C. dubliniensis* and *C. gattii* need to be carefully screened and tested before being used in co-culture fermentations of cereal-based beverages as they are main agents of human yeast infections such as blood stream and mucosal yeast infections (Johansen *et al*., 2019).

4.6 Molecular characterization of isolates

4.6.1 *Lactobacillus spp*.

The PCR amplicons were confirmed to be *Lactobacillus spp*. with approximately 250 bp based on the methodology adopted. Plate 4 shows typical results of the PCR amplifications. After sequencing, some isolates had similarity scores ranging from 98 to 100 percentage identity with *Lactobacillus* species in the GenBank (NCBI). A phylogenetic analysis grouped the isolates from this study into two important *Lactobacillus spp*. with 100% frequency. These were *L. plantarum* and *L. brevis* (Fig. 5).

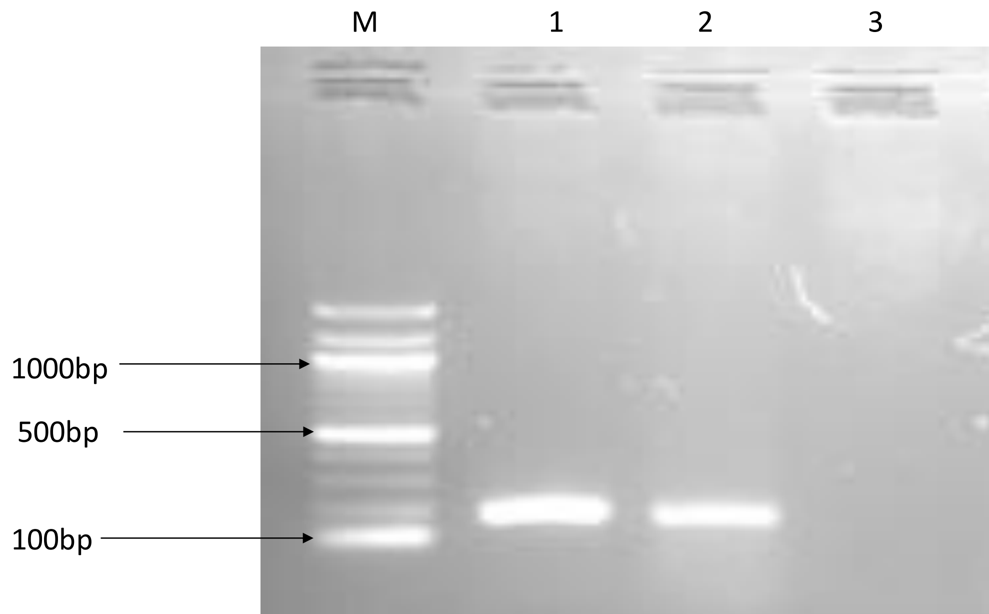

Plate 4: PCR Amplicons from *Lactobacillus* isolates. Lane M: 100 bp DNA ladder, lane 1and 2 are representative *Lactobacillus* isolates, lane 3: Negative control. The amplicons have approx. 250bp (Ségolène *et al*., 2002)

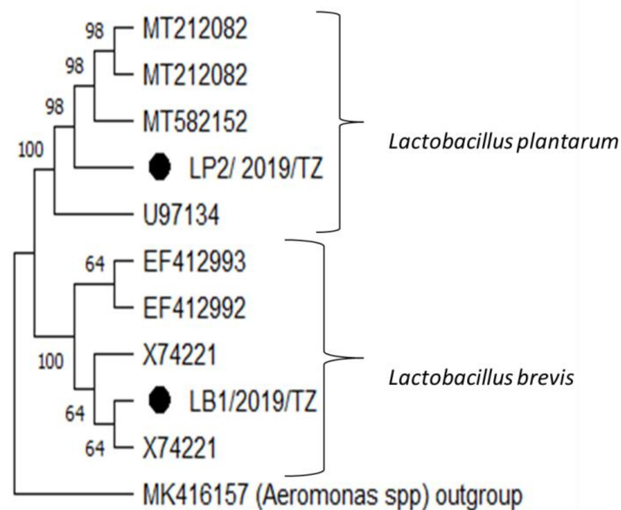

Figure 7: Phylogenetic tree of representative *Lactobacillus* isolates from this study (black circle) and closely related taxa from the GenBank. The tree was generated using Neighbor-Joining method (p-distance model), bootstrap values expressed as percentages of 1000 replications. *Aeromonas veronii* served as an out-group

4.6.2 *Candida spp*. (yeasts)

The PCR results established that the isolates were yeasts as the amplicons had approximately 450 – 800 bp based on the methodology adopted. Typical results of the PCR amplicons are shown in Plate 5.

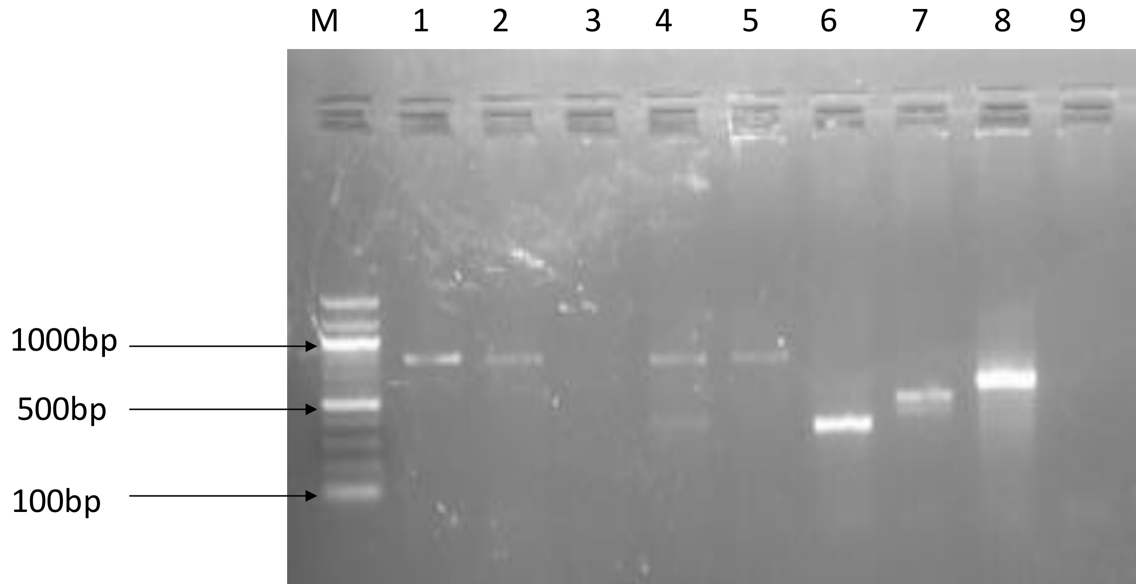

Plate 5: PCR Amplicons from yeast isolates. Lane M: 100 bp DNA ladder, lane 1 – 8 are representative yeast isolates from cereal-based beverages, lane 9 is a negative control

Upon blasting, it was revealed that the yeast sequences from this study were identical to the yeast sequences of two genera in the GenBank (NCBI) with similarity scores ranging from 99-100%. A phylogenetic analysis placed yeast sequences in this study into two respective yeast species with reference to sequences from the GenBank namely; *Candida tropicalis* and *Pichia kudriavzevii* (Fig. 8). However, *P. kudriavzevii* was not among the yeast species identified during biochemical analysis but only after sequencing the PCR products. Such result is inevitable since biochemical tests may give similar identity to microbes which show similar characteristics though they might be different species (Johansen *et al*., 2019). *Pichia. kudriavzevii* (formerly *Issatchenkia orientalis*) has been reported in 60% of the 43 indigenous fermented foods and beverages surveyed in sub-Saharan Africa (Johansen *et al*., 2019).

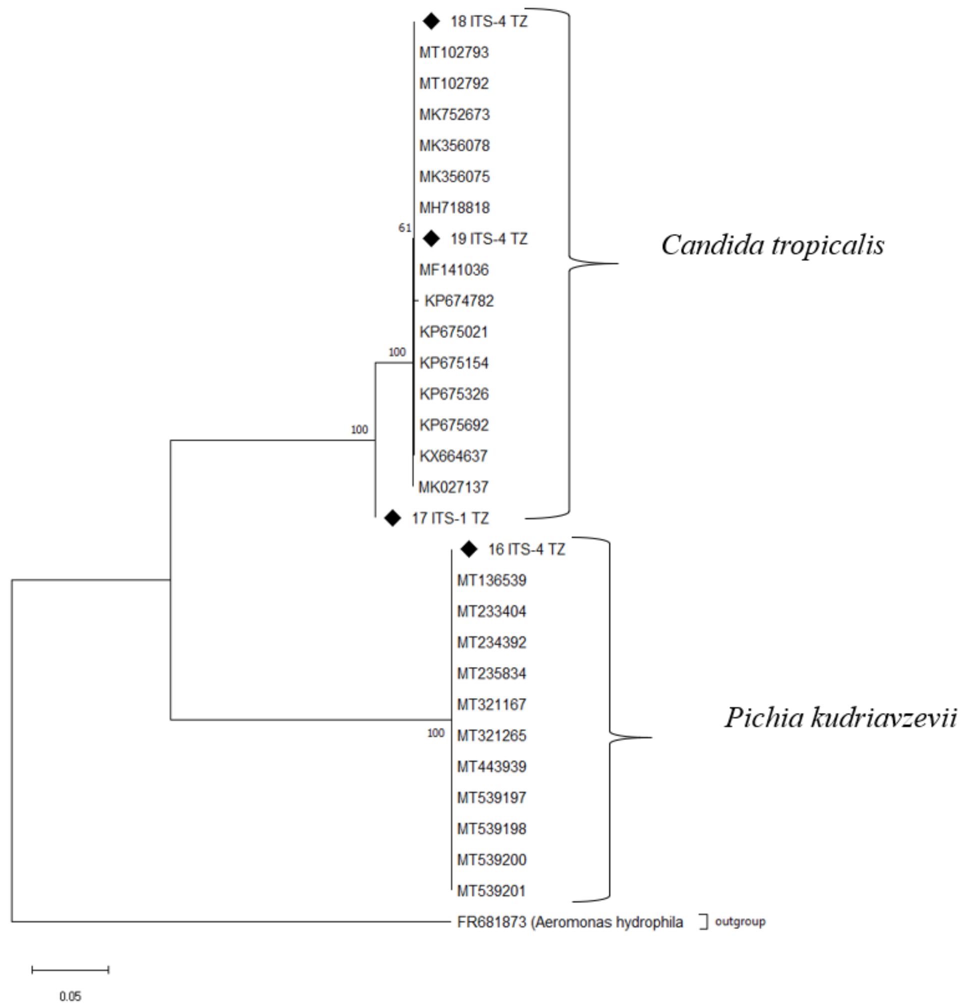

Figure 8: Phylogenetic tree of representative yeast isolates from this study (black diagonal) and closely related taxa from the GenBank. The tree was generated using Neighbor-Joining method (p-distance model), bootstrap values expressed as percentages of 1000 replications. *Aeromonas hydrophila* served as an out-group

4.7 Probiotic cereal-based beverages production with selected starter cultures

The starter cultures of LAB selected for the controlled fermentations of the four-probiotic cereal-based beverages were *L. brevis* for *Togwa*, *P. pentosaceus* for *Kindi* and *L. plantarum* for both *Kimpumu* and *Mbege*. These were used for optimization of fermentation processes, the quality and safety of the final products. The production of probiotic cereal-based beverages is a pioneering initiative that opens an avenue for scaling up and commercialization of the beverages for consumption not only in the villages but also in urban and peri urban areas. In the 24 h time interval of the controlled fermentations; *Kimpumu* and *Mbege* seem to have lower pH values compared to Kindi and Togwa. This shows that there was more acidification in *Kimpumu* and *Mbege* due to production of higher amounts of lactic acid and other organic acids

which lowered their pH values than in *Kindi* and *Togwa* in which the acidification was slow resulting to higher pH values. Correspondingly, *Kimpumu* and *Mbege* had higher viable cell counts; 11 log cfu/mL than *Kindi* and *Togwa*; 10 log cfu/mL within the same time interval because of the differences in microbial activity, metabolites produced and pH values (Gupta & Bajaj, 2017; Kumar *et al*., 2015). It is obvious that *L. plantarum* which is the probiotic culture in both *Kimpumu* and *Mbege* is a high acid producer and favours high acidity (lower pH) for its proliferation (Mukisa *et al*., 2017; Oguntoyinbo & Narbad, 2015) compared to *P. pentosaceus* and *L. brevis* present in *Kindi* and *Togwa*, respectively. *L. plantarum* and *P. pentosaceus* are homofermentative during fermentation while *L. brevis* is heterofermentative (Bennani *et al*., 2017; Kohajdová, 2016). Thus, high amount of lactic acid and other acidic metabolites produced play an important role in the proliferation of some species of LAB (Mukisa *et al*., 2017) during fermentation of the cereal-based beverages described in the succeeding sections.

Angelov *et al*. (2006) developed an oat-based probiotic drink using *L. plantarum* as a starter culture under controlled fermentation at 37 °C for 8 h. The controlled fermentation helped to ensure that the pH of the final probiotic oat drink was in the range 4.0 – 4.5, viable cell count of approximately 10 log cfu/mL and beta-glucan content in the oat mash of 0.31 –0.36%. Controlled fermentation ensured fast fermentation, avoidance of microbial contamination, higher viable cell counts and appropriate content of beta-glucan in the drink (Angelov *et al*., 2006). In another study, controlled fermentation at 37 °C of rice mixed with cassava flour and inoculated with either single culture of *L. plantarum*/*L. acidophilus* or in co-culture with the yeast strain *Toluraspora delbrueckii* produced a probiotic drink in 36 h of fermentation with viable cell count above 7.5 log cfu/mL. Fermentation beyond 36 h resulted to decreased viable cell count (Freire *et al*., 2017). Thus, 36 h were a suitable length of time for the substrate fermentation. It helped to reduce production costs and increased productivity of the process (Freire *et al*., 2017). Generally, controlled fermentation includes selection of efficacious microbial strain or strains, suitable substrate, known fermentation temperature and the length of the process. This ensures optimization of the fermentation process (faster, shorter time) and production of the cereal beverage that is of high organoleptic quality, safety and microbial stability (Freire *et al*., 2017; Mugula *et al*., 2003b; Mukisa *et al*., 2016; Navarrete-Bolaños, 2012). Thus, strain or strains selection for a particular fermented product forms the basis for a controllable, predictable and efficient fermentation process (Navarrete-Bolaños, 2012).

4.7.1 Probiotic Togwa

Fresh *Togwa* with probiotic characteristics resulted after 24 h-controlled fermentation (Fig. 5). It was a resultant beverage with suspended particles of maize and millet grains that had a sweet-sour smell, slightly sweet-sour taste and pale brown; a color derived from its constituents. The fresh beverage exhibited a mean viable cell count of 5.35 x 10^{10} cfu/mL and a mean pH value of 5.58. This is the first study to report preparation of *Togwa* with probiotic characteristics under controlled fermentation conditions. Similar controlled fermentation was employed to study the ability of LAB and yeasts isolated from native *Togwa* to ferment the maize-sorghum gruel used as a substrate in *Togwa* production (Mugula *et al*., 2003b). The tested *spp*. of LAB was able to lower the pH to 3.24 - 3.49 and increased the acidity within 24 h with viable cell count of about 10^9 cfu/mL. In the same time interval, yeast *spp*. lowered the pH to 3.57 - 4.81 and increased the acidity of the gruel. With controlled fermentation, it was possible to eliminate the pathogenic Enterobacteriaceae and end up with a fermented cereal-based beverage with acceptable organoleptic quality, safety and stability (Mugula *et al*., 2003b). In another study, *Enturire*; a spontaneously produced traditional sorghum-based beverage in Uganda that does not guarantee quality and safety; was produced under controlled fermentation using pure starter cultures of LAB and yeast (*L. plantarum, W. confusa and S. cerevisiae*) resulting to an acceptable *Enturire* in a short time (Mukisa *et al*., 2016). Aka *et al*. (2014) has reported application of controlled fermentation using pure starter cultures of LAB and yeasts to produce cereal-based beverages such as *Pito* (native to Nigeria and Ghana) and *Tchoukoutou* (native to Benin, Togo), which were all potentially probiotic.

Controlled fermentation is essential for *Togwa* production because it optimizes the process, makes the process controllable, predictable and efficient. Acidification of the beverage is faster and the desired pH value preferably 3.8 or lower is reached within a shorter time (Mugula *et al*., 2003b; Navarrete-Bolaños, 2012). The end product is obtained within a short span of time with high organoleptic quality, safety and stability compared to spontaneous fermentation. Moreover, controlled fermentation provides *Togwa* with macro and micro-nutrients such as simple sugars and minerals like Fe, Zn, Ca, Mn (Kohajdová & Karovičová, 2007; Nyanzi & Jooste, 2012). It provides the B-group vitamins, specifically folic acid which is important for developing the neural tubes in infants and help to prevent some diseases such as coronary heart diseases, stroke and certain cancer (Angelov *et al*., 2018; Lara-Hidalgo *et al*., 2017). Probiotic *Togwa* has the potential; like other probiotic products, to provide health, preventive and

curative benefits to humans such as enhancement of immunity, prevention of gastrointestinal disorders, lactose intolerance, allergy, high blood pressure (BP), infantile diarrhea, acute and induced diarrhea, and life style diseases such as obesity, cardiovascular disorders, diabetes and cancer (Markowiak & Ślizewska, 2017; Panghal *et al*., 2018; Ranadheera *et al*., 2017). Unlike spontaneously produced *Togwa* that has a mixture of probiotic and non-probiotic microflora (possibly pathogens, fungi and molds), compromised quality, safety and stability; probiotic *Togwa* produced by carefully selected microbes under controlled fermentation like the one in this study; has potentially acceptable quality attributes, safety and stability (sections 4.9, 4.10, and 4.11). The production of probiotic *Togwa* in this study is a pioneering initiative that opens an avenue for scaling up and commercialization of the beverage so that household producers (mostly women) and SMEs produce the drink for consumption not only by people of lower social status in the villages but also those in urban and peri-urban areas. They will be able to earn more incomes and improve their livelihood and simultaneously providing health benefits to the consumers. This will happen when the outcomes of this study are further validated empirically. Spontaneously fermented *Ogi* (Nigerian fermented maize, millet, or sorghum-based beverage) was improved to probiotic *Dogik* (using LAB starter culture) and scaled up as it had beneficial effects against diarrhea causing bacteria (Blandino *et al*., 2003b; Soro-Yao *et al*., 2014).

4.7.2 Probiotic Kindi

Probiotic *Kindi* in fresh form was obtained following 24 h - controlled fermentation (Fig. 3). The beverage was whitish-pale brown, had a slightly sweet-sour taste, sweet-sour smell and suspended particles of maize and millet grains. The pH of the fresh beverage was 5.33 and its viable cell count was 1.45 x 10^{10} cfu/mL.

As is with the preceding beverage; this study is the first to report the preparation of *Kindi* with probiotic characteristics employing controlled fermentation. Spontaneously fermented *Kindi*; as the local beverage, has never been studied before, probably due to its consumption by mainly rural communities. But with the current revelation of being produced as a probiotic drink, it is anticipated that more people will go for it in order to get health benefits associated with the probiotic bacteria it contains. The composition of probiotic *Kindi* is similar to that of *Kwete* (Uganda) based on the substrate mix of cereals and genus of LAB used although the latter is spontaneously produced in co-culture of LAB and yeasts (Aka *et al*., 2014). The use of pure culture, known fermentation time and temperature in *Kindi* production makes the fermentation

process controllable so that it is possible to predict the viable cell count of the pure culture employed (for probiotic characteristics), the range of the pH change in the product as a result of the production of lactic acid and other known metabolites produced by the culture; and the quality, stability and safety of the end product. Therefore, when produced under optimized conditions; *Kindi* has the probiotic potential by carrying large populations of probiotic LAB above the threshold dose of 10^6 cfu/mL and thus impart health benefits to consumers such as prevention and cure of acute diarrhea, diabetes, CVD and bowel disorders.

4.7.3 Probiotic Kimpumu

Probiotic *Kimpumu* was the result of 24 h - controlled fermentation of cooked finger millet malt flour. This beverage had a sweet-sour taste, sweet-sour smell, was pale brown with suspended particles of millet grains. The viable cell counts of the fresh probiotic *Kimpumu* was 1.49 x 10^{11} cfu/mL and its pH 5.2.

Probiotic *Kimpumu* is being produced for the first time in this study. It has not been produced and studied before possibly because it was prepared by local people as spontaneously fermented drink and consumed only in rural communities. However, being produced as a probiotic cereal-based beverage during this study; it goes a long way as one of the fermented cereal-based beverages that provides consumers with nutrients from fermented cereals as well as impart the health benefits associated with probiotic bacteria. Probiotic *Kimpumu* can be likened to *Malwa* (Uganda), *Mangisi* (Zimbabwe) and *Masvusvu* (Zimbabwe) in terms of the substrate used and genus of fermenting LAB though the later are all spontaneously prepared with a consortia of LAB, yeasts and molds (Aka *et al*., 2014). The probiotic *Kimpumu* is produced under controlled fermentation process as such it contains known pure culture of a bacterium which ferments the beverage at known temperature and duration of time. Its activity in the given cereal substrate is known and the viability has been shown to be high enough to surpass the minimum dose required for probiotic foods. Production of probiotic *Kimpumu* under the given conditions guarantees that the end product falls within a predictable pH range, which ensures quality organoleptic attributes, shelf stability and safety. Thus, when produced under optimized conditions; the beverage contains LAB beyond the threshold dose of 10^6 cfu/mL and consequently exhibit probiotic characteristics, which are desirable for deliverance of health benefits to consumers.

4.7.4 Probiotic Mbege

This beverage was obtained as the end product of 24 h – controlled fermentation of a mixture of *Nyalu* and *Mso*. The drink is reddish brown with suspended particles of millet grains. It had a sweet-sour taste, sweet-sour smell, and mean viable cell count of 1.38 x 10^{11} cfu/mL and pH 4.93, respectively when freshly produced.

Probiotic *Mbege* has rarely been produced before in Tanzania. Again, this is the first study to come up with such a product. In a previous study, Kubo and Kilasara (2016) examined various techniques used in the production of local spontaneously fermented *Mbege* in North Eastern and Southern parts of Tanzania and compared their production techniques. It was concluded that in both areas the production techniques were similar (Kubo & Kilasara, 2016). Moreover, no microbiological enquiry was done. Controlled fermentation is expected to revolutionize the way spontaneously fermented *Mbege* is locally produced in order to take advantage of the innovations derived from this study. Use of pure culture of LAB during production of *Mbege* is the first step to ensure that the fermentation process is predictable, reliable and efficient (Freire *et al.*, 2017; Ivan *et al.,* 2017). Controlled fermentation in *Mbege* production ensures that the process is faster, desired pH achieved in a shorter time and the shelf life and safety of the product is guaranteed (Setta *et al*., 2020). Optimization of fermentation conditions can make the beverage potentially health giving like any other probiotic product. The potential health-giving properties include preventive and curative properties for many endemic and lifestyle diseases such as acute diarrhea, gastro-intestinal disorders, lactose intolerance, cancer, obesity, diabetes, high blood pressure, and CVD.

4.8 Probiotism of the cereal-based beverages

Figures 9 and 10 represent the results for mean viable cell counts and pH for the four cereal-based beverages after 48 h of controlled fermentations. Assessment of probiotism for *Togwa, Kindi, Kimpumu* and *Mbege* indicated that the viable cell counts and pH values at all fermentation times were 1 x 10^{11} cfu/mL with pH value 4.96 for *Togwa*; 5 x 10^{10} cfu/mL with pH value of 4.83 (Kindi); 2 x 10^{11} cfu/mL with pH value of 4.72 (*Kimpumu*) and 2 x 10^{11} cfu/mL with pH value of 4.59 (*Mbege*). Within the given time range of 0 - 48 h; all the three cultures showed a mean viable count of 1.0 x 10^{11} cfu/mL and a mean pH value of 4.77, respectively. The values for viable cell count and pH for the respective beverages are significant different at $p < 0.001$.

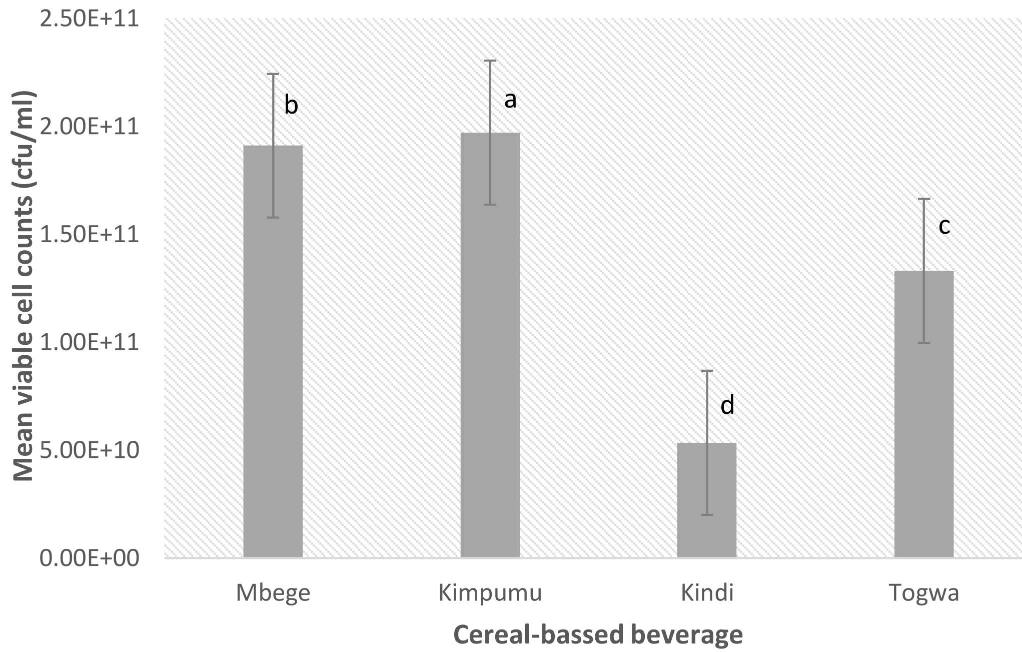

Figure 9: Viable cell counts of fermented cereal-based beverages, error bars present Mean ±SD, n = 48. The grey bars represent viable cell counts

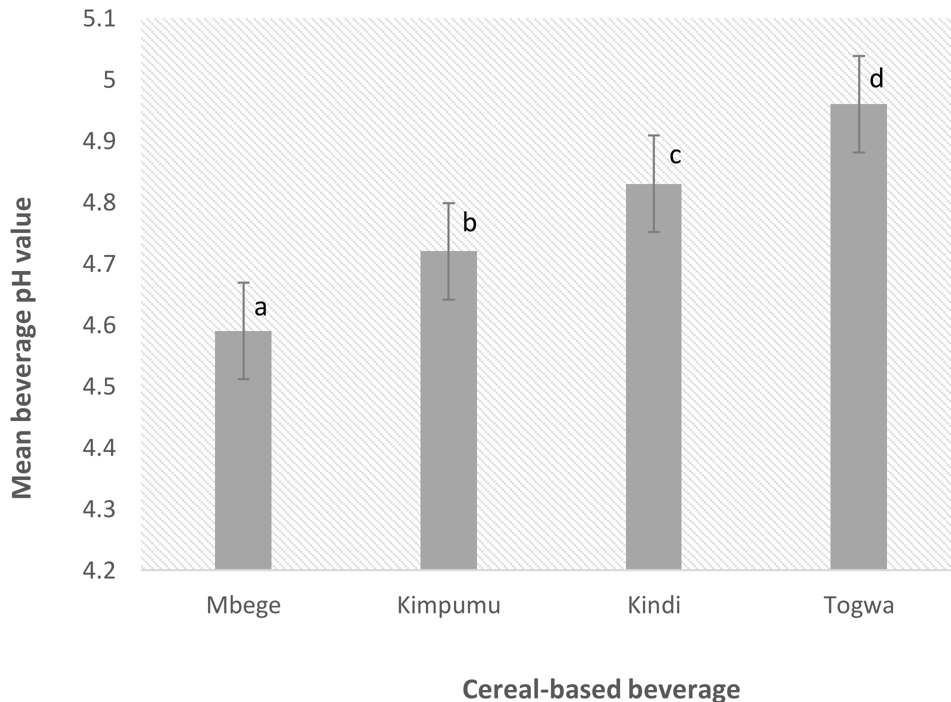

Figure 10: The pH values of fermented cereal-based beverages, error bars present Mean ±SD, n = 48. The grey bars represent pH values

Empirical evidence suggests that the fermented cereal-based beverages prepared in this study were potentially probiotic. All fermented beverages prepared had the viable cell count of 11 log cfu/mL or 10^{11} cfu/mL, which is far more above the threshold value of 10^{6} cfu/mL

(Enujiugha & Badejo, 2017; Mokoena *et al*., 2016) for probiotic foods. The pH of the respective beverages is in the range of 4.0 – 5.0 that suggests that they are moderately sour and can easily be consumed (Mukisa *et al*., 2016; Salmerón *et al*., 2015). Normally, fermented cereal-based beverages are still consumable at the minimum pH value of 3.5 and above (Mugula *et al*., 2003; Salmerón *et al*., 2015). The relative difference in the viable cell counts of the fermented cereal-based beverages produced under controlled conditions is attributed to activity and specificity of the probiotic bacterium in each of them, the substrate composition in each product in terms of the nutrients required by respective bacteria, the end metabolic products released by each bacterium and particularly production of lactic acid, and the pH change as a result of increase in the production of lactic acid. In this study, an increase in viable cell counts was observed relative to the fermentation time span (Fig. 11) whereas, a decrease in pH was induced by aggravated release of the lactic acid which is the main end product of the fermentation process (Fig. 12). Lactic acid bacteria are acidophilic and tend to increase exponentially in acidic environment (Hammes & Hertel, 2009; Mukisa *et al*., 2017), however, this increase is limited by too low pH such as that below 3.5 (Mukisa *et al*., 2016).

The controlled fermentation results were similar to those reported by Angelov *et al*. (2006) who developed an oat-based probiotic beverage in 8 h fermentation at 37 °C using probiotic *L. plantarum* as a starter culture and the end product had viable cell count of 7.5 x 10^{10} cfu/mL and pH of 4.0 - 4.5. In another study, Gupta and Bajaj (2017) reported that development of probiotic fermented oat-flour beverage (PFOF) resulted to a probiotic drink with viable cell count of 10^{14} cfu/mL after 72 h of fermentation at 37 °C. Salmerón *et al*. (2015) developed cereal-based beverages from three cereal substrates; malt, barley, and oat through a 10 h fermentation at 37 °C using mono-culture strains of *L. plantarum, L. acidophilus*, and *L. reuteri* that resulted to potential probiotic cereal beverages with viable cell populations ranging from 7.8 - 8.2 log cfu/mL. The preceding investigations exhibit the suitability of cereal substrates as delivery vehicles for probiotics. This can lead to a conclusion that cereals as substrates support to a greater extent the growth of LAB during cereal fermentations. Therefore, there is a huge potential in utilizing fermented cereal-based beverages as delivery media of probiotics to human being.

Further results of the 48 h – controlled fermentations of cereal-based beverages show that the viable cell counts increase with increase in fermentation times whereas conversely, the pH values decrease with increase in fermentation times (Figure 11 and 12).

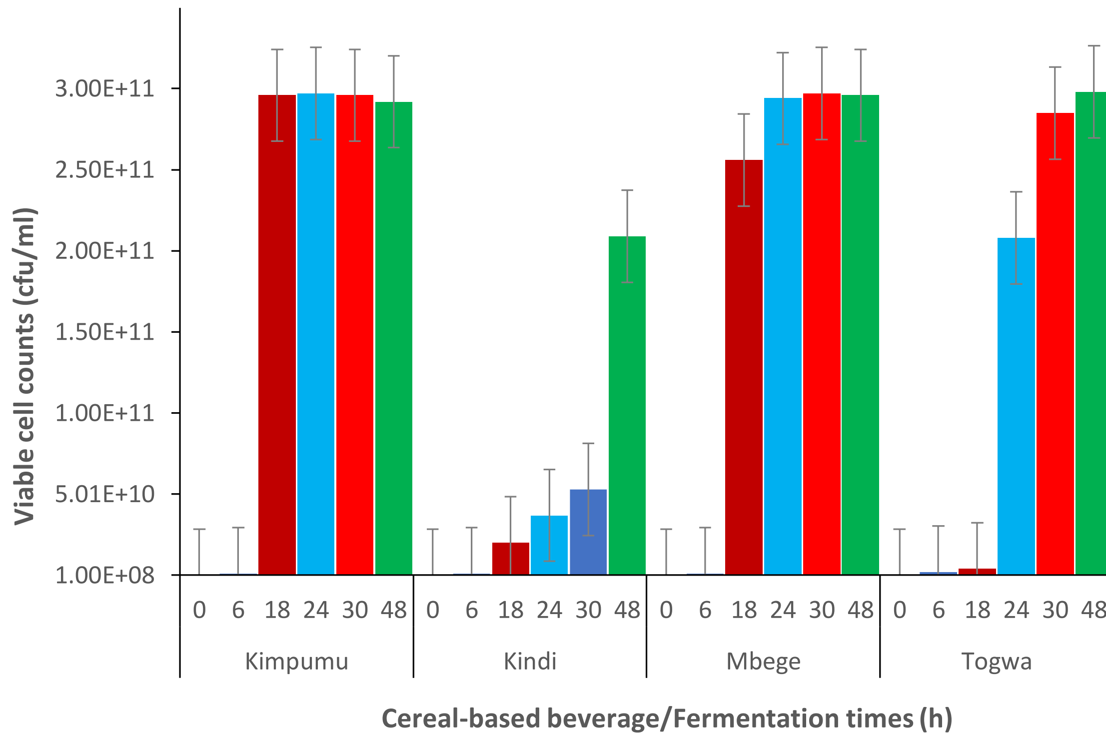

Figure 11: Changes in viable cell counts in fermentations of cereal- based beverages, error bars present Mean ±SD, n = 48. The colored bars represent viable cell counts

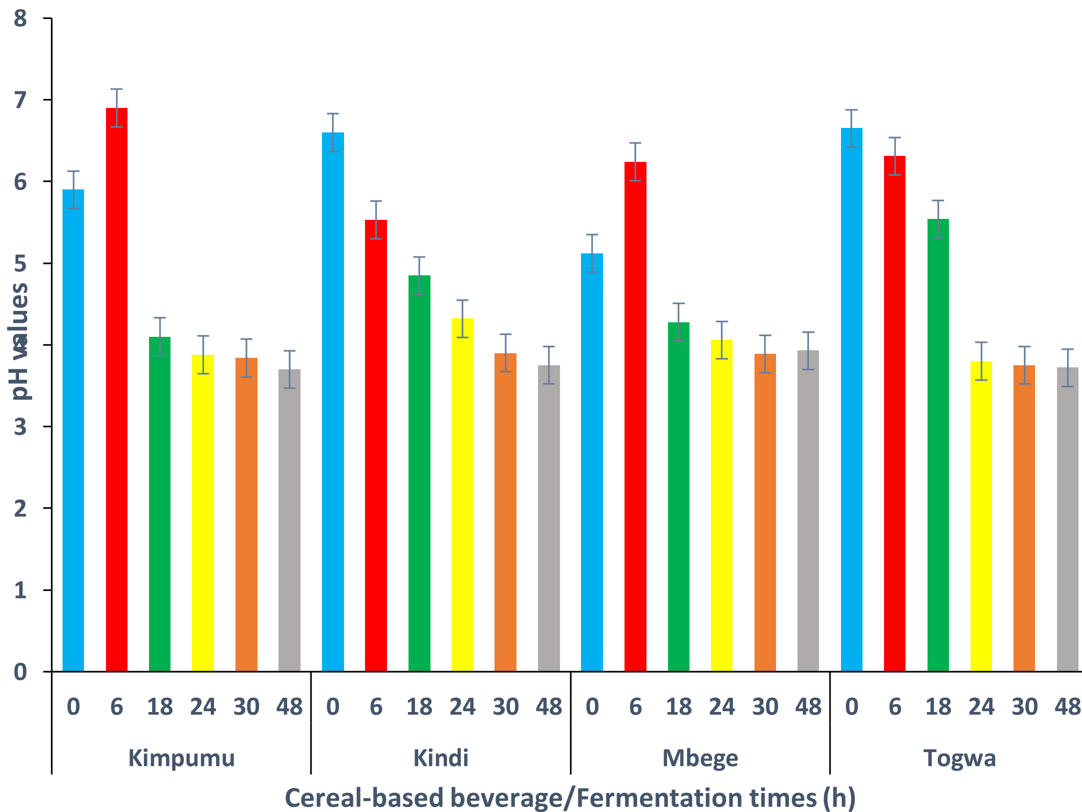

Figure 12: The pH values of cereal-based beverages as related to fermentation times, error bars present Mean ±SD, n = 48. The colored bars represent pH values

There is also an inverse relationship between the pH values of the cereal-based beverages and viable cell counts (Fig. 13).

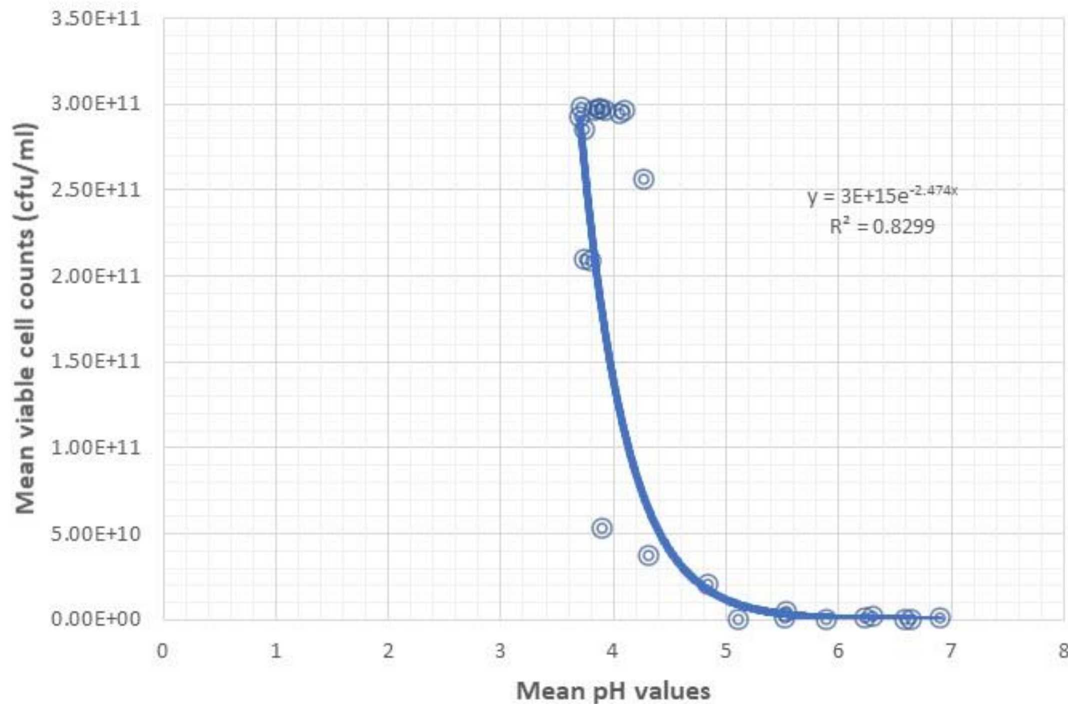

Figure 13: Association between viable cell counts and pH values during controlled fermentations of cereal-based beverages

The inverse relationship between pH and viable count (Fig. 13) is due to the fact that the growth of LAB is dependent on the amount of acid produced in the substrate and the corresponding reduction in pH (Hammes & Hertel, 2009). At the beginning of the fermentation process there was very small amount of acid present yet (Fig. 12) as the pH was around 6 -7 and the corresponding increase in bacterial cells was very low since the pH was also still higher (Figure 11 and 12). However, this association does not apply to the entire pH range during fermentation. It is only applicable from the start of fermentations to the limiting pH of approximately 3.7 as shown in Fig. 13.

4.9 Acceptance of the probiotic cereal-based beverages

Results of the acceptance test of the fermented probiotic cereal-based beverages are depicted in Table 9.

Table 9: Acceptance mean scores of the probiotic cereal-based beverages

Cereal-beverage	Appearance	Color	Taste	Aroma	Texture	Overall acceptability
Togwa	2.06^{a}	2.00^{a}	1.86^{a}	1.94^{a}	2.06^{a}	2.04^{a}
Kindi	2.12^{a}	2.16^{ab}	2.74^{b}	2.24^{b}	2.54^{b}	2.24^{ab}
Mbege	2.16^{a}	2.18^{ab}	1.92^{a}	1.74^{a}	2.20^{a}	1.96^{a}
Kimpumu	2.60^{b}	2.30^{b}	2.68^{b}	2.26^{b}	2.72^{b}	2.42^{b}

Note: Results are mean scores ± SD of the acceptance test (n=50). Values with different superscript letters within the same column are significantly different at $p < 0.001$ level of significance. Acceptability was evaluated based on the 7-point hedonic scale; from 1 (like extremely) to 7 (dislike extremely).

Similarly, results for Just-about-right scale (JAR) with the centre point choice (Just-about-right) percentages for panellists' reactions for specific product attributes are summarised in Table 10. The JAR - test measures the consumers' reactions to specific product attributes and shows the product attributes which need adjustments or optimisation to meet consumer preferences.

Table 10: Percentage results for JAR scale on the product attributes

Attribute (%)/ Cereal beverage	Sweetness	Acidity	Taste	Aroma	Color	Texture
Togwa	80	66	68	84	90	62
Kindi	44	54	48	80	68	48
Mbege	88	76	66	90	92	66
Kimpumu	58	52	50	82	86	26

Further results on the acceptance test indicated that the most preferred probiotic cereal-based beverage was *Togwa* (52% of the respondents) followed by probiotic *Mbege* (44% of the respondents). The majority of respondents (94%) were interested in buying probiotic *Togwa* and *Mbege* though 56% and 70% were interested in buying probiotic *Kimpumu* and *Kindi*, respectively.

The acceptability test outcomes given in Table 9 above show that each of the four-probiotic cereal-based beverages had good and promising quality attributes (hedonic scale score 2) in terms of appearance, color, taste, aroma, texture as well as overall acceptability. Salmerón *et al*. (2015) reported equivalent acceptability of three probiotic cereal beverages; barley (with *L. acidophillus)*, malt (with *L. reuteri*) and oat (with *L. reuteri*) by having similar scores. It has also been reported that fermented cereal beverages with mid pH values; as observed in this study, are more acceptable (Salmerón *et al*., 2015). The findings in Table 10 (based on JAR scale) give the implication that the sweetness of the probiotic beverages is appropriate (above 50%) with the exception of probiotic *Kindi*, which needs further adjustment or optimisation. Acidity, aroma and color of the probiotic beverages were quite right (above 50%) implying

that the attribute levels attained in this study are suitable when producing such beverages for home consumption or sale. The taste and texture of probiotic *Kindi* also needed some adjustment or optimization since only 48% of the test panellists indicated that such characteristics are just-about-right. On the other hand, probiotic *Kimpumu* had to some extent been well evaluated, however the percentage of panellists asserting its texture as just appropriate was very low, possibly because of having higher score (hedonic score 2.72) in textural properties than the rest of the beverages (Table 9).

Cereal-based probiotic *Togwa* was the most preferred of all prepared cereal-based probiotic beverages in this study simply because it showed the lowest score values of all the quality attributes (Table 9). Similarly, probiotic *Mbege* was likewise more preferred as it has score values significantly comparable to *Togwa*. More preference of consumers to probiotic *Togwa* and *Mbege* than the remaining tested probiotic beverages is due to their much better organoleptic attributes than the rest (Table 9).

4.10 Stability of beverages

4.10.1 Storage at ambient temperature

Storage of the four probiotic beverages at ambient temperature (25 °C) for five days indicated that the pH values of the beverages were significant different ($p < 0.001$). In the case of viable cell counts; *Kimpumu* and *Mbege* showed no significant difference (They had the same probiotics count), and the cell counts for these two beverages were significant different from the rest (*Togwa* and *Kindi*). *Kimpumu* and *Mbege* exhibited the highest viable cell counts whereas *Togwa* and *Kindi* displayed lower values (Fig. 14). *Kimpumu* showed the lowest pH value of 3.69 while *Togwa* displayed the highest pH value of 3.97 (Fig. 15).

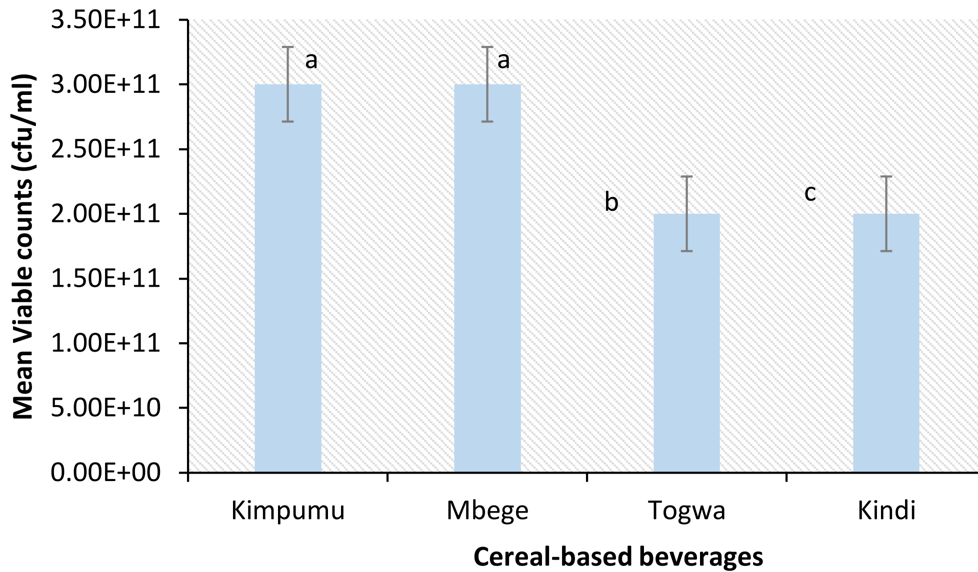

Figure 14: Mean viable cell counts of cereal-based beverages stored at ambient temperature (25 °C) for 5 days. Error bars represent Mean ±SD, n = 48. The bars stand for viable cell counts

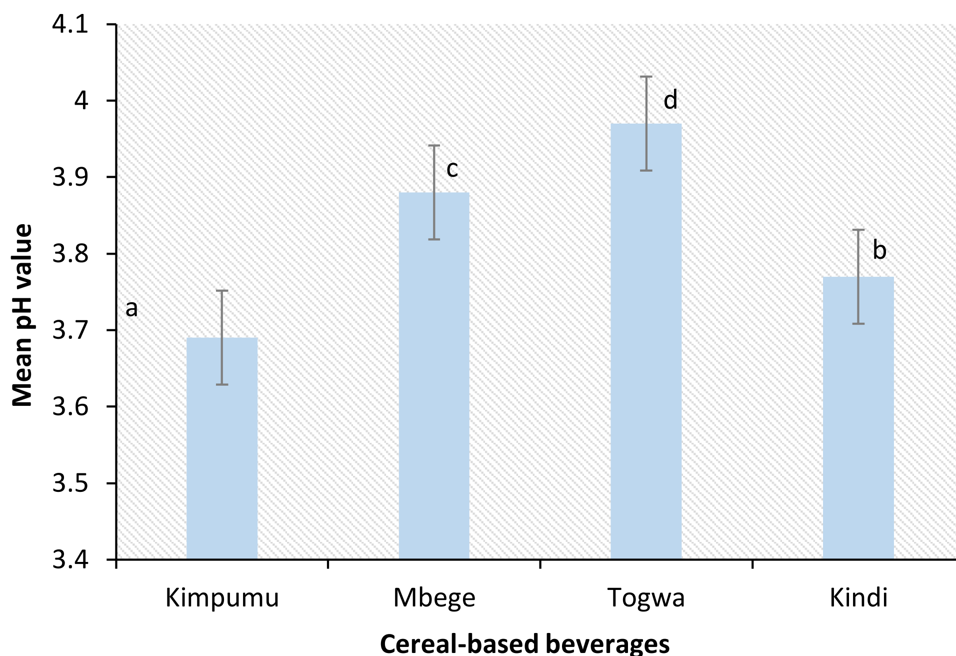

Figure 15: Mean pH values of cereal-based beverages stored at ambient temperature (25 °C) for 5 days. Error bars represent Mean ±SD, n = 48. The bars stand for pH values

Further results on storage of the potential probiotic products for 5 days at 25 °C show that the viable cell counts at all storage times are above the threshold value of 1 x 10^6 cfu/mL for probiotic products (Fig. 16). The pH values of the products at all storage times are in the range 3.40 – 4.64 with a mean pH value of 3.83 (Fig. 17).

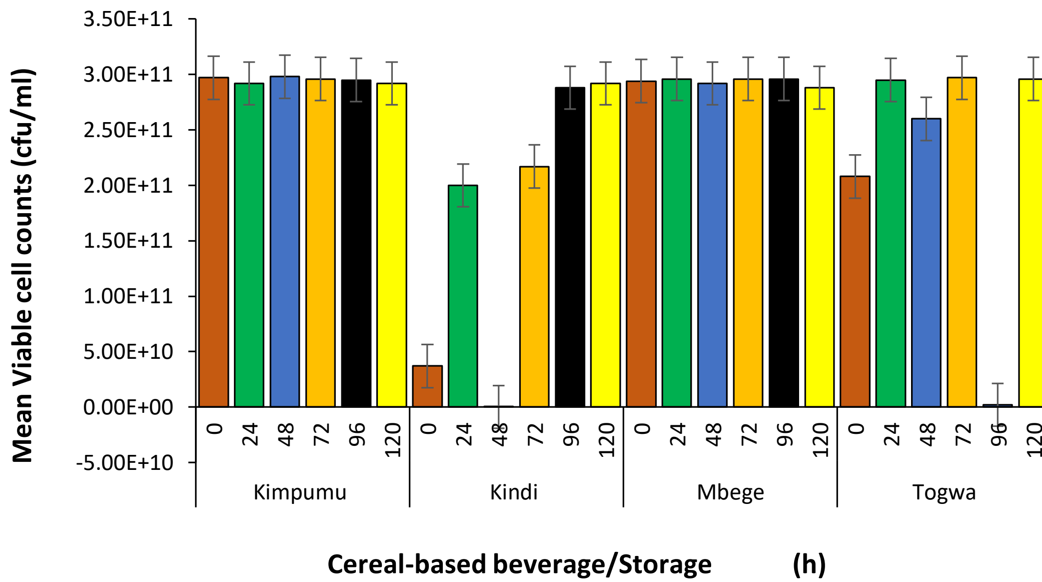

Figure 16: Viable cell counts at different storage times at 25 °C, error bars represent Mean ±SD, n = 48. The colored bars represent mean viable cell counts

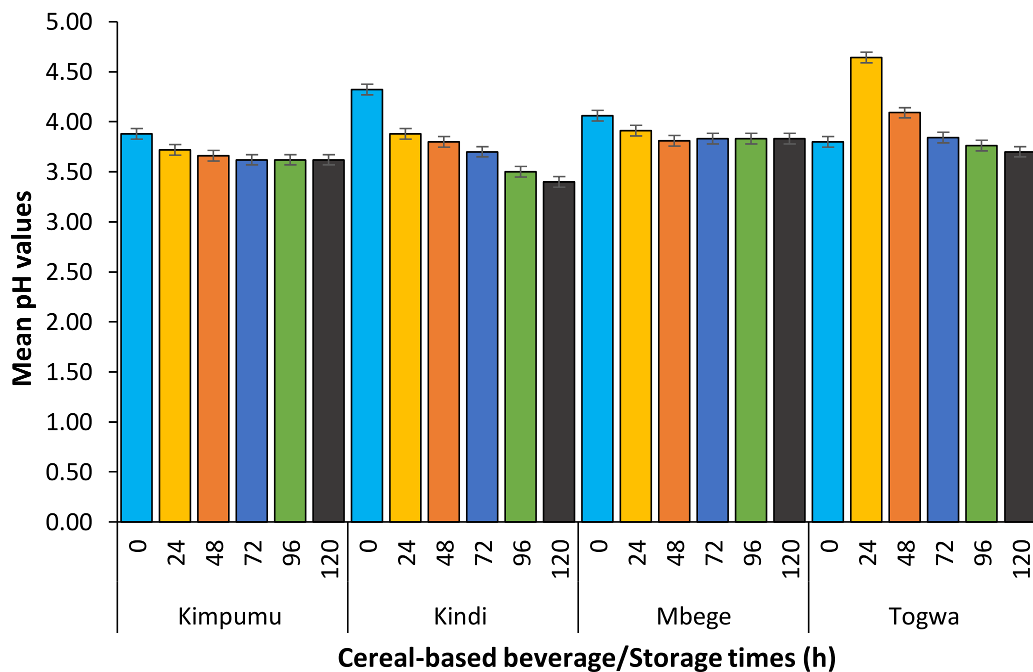

Figure 17: The pH values at distinct storage times at 25 °C, error bars represent Mean ±SD, n = 48. The colored bars represent mean pH values

The results above clearly indicate that probiotic *Togwa, Kindi, Kimpumu* and *Mbege* prepared using pure probiotic starter cultures under controlled fermentation can maintain their probiotic and sensory qualities at ambient temperature (25 °C) for 5 days with an overall viable cell count of 2.0 x 10^{11} cfu/mL and the pH value of 3.83 (Figure 16 and 17). This pH value is above the minimum pH value of 3.5; below which most cereal-based beverages of local origin become

exceedingly sour and unacceptable for consumption (Mugula *et al*., 2003b; Mukisa *et al*., 2016; Salmerón *et al*., 2015). The safety of the cereal beverages was ensured by absence of Enterobacteriaceae at the end of 5 days (section 4.11).

The growth of *L. plantarum* in probiotic *Kimpumu* and *Mbege* was almost instantaneous at ambient temperature storage (Fig. 16). At every storage span, the viable cell counts had reached the maximum of about 3.0 x 10^{11} cfu/mL and the pH was in the range of 3.62 – 4.06. In the case of *Kindi* and *Togwa*, the growth of *P. pentosaceus* and *L. brevis* was not rapid initially but later increased and then decreased before reaching the maximum of about 3.0 x 10^{11} cfu/mL at the end of the 5 days with a pH range of 3.40 – 4.64 (Figure 16 and 17). Most of the locally available fermented cereal beverages are spontaneously fermented resulting to products with unknown probiotic quality and pH values and this is the basis for having shorter shelf-lives of at most two days (Aka *et al*., 2014; Mokoena *et al*., 2016). Di Stefano *et al*. (2017) has shown that storage at ambient temperature of a probiotic water-based millet product (8% pre-treated millet in water, 5% sucrose) maintained the stability of the product for 5 days with probiotic viable cell count of 9 log cfu/mL and a pH of 3.9. In another similar study by Gupta and Bajaj (2017); a probiotic oat beverage indicated viability of more than 10^6 cfu/mL for *L. plantarum* which remained higher after three weeks of storage at 25 °C. The difference in the growth pattern and the pH decrease as observed in this study and the previous ones suggest the fact that survival of a probiotic bacterium differs from other probiotics depending on the composition of the supporting matrix, the environmental conditions, the metabolites it produces and the characteristics of the bacterium itself (Gupta & Bajaj, 2017; Kumar *et al*., 2015). The promising results of probiotic beverage stability at ambient temperature for 5 days open up an avenue for small-scale and medium scale producers of fermented cereal-based-beverages in Tanzania to seize the opportunity, improve their fermentation and storage infrastructure and scale-up the outcomes of this research. This will boost their incomes through extended shelf lives of the products (5 days instead of 2), improve the health of the majority of people in the country and the national economy as a whole. However, the opportunity for improving the cereal-based beverages can only happen when such results are further validated.

4.10.2 Storage at refrigeration temperature

The results of the viable cell count of probiotic *Kimpumu, Kindi, Togwa* and *Mbege* preserved at refrigerated temperature (4 °C) for 28 days are as shown in Fig. 18. A non-significant difference ($p < 0.001$) in mean viable cell counts was observed among *Kimpumu, Kindi* and

Mbege. However, the viable cell count of *Togwa* was significantly different ($p < 0.001$) from the rest. The former trio displayed the highest viable cell counts of 2 x 10^{11} cfu/mL while the later had viable cell count of 1 x 10^{11} cfu/mL (Fig. 18). On the other hand, the pH values of probiotic *Kindi* and *Mbege* were not significant different ($p < 0.001$) but there was significant difference in pH values between probiotic *Kimpumu* and *Togwa* (Fig. 19).

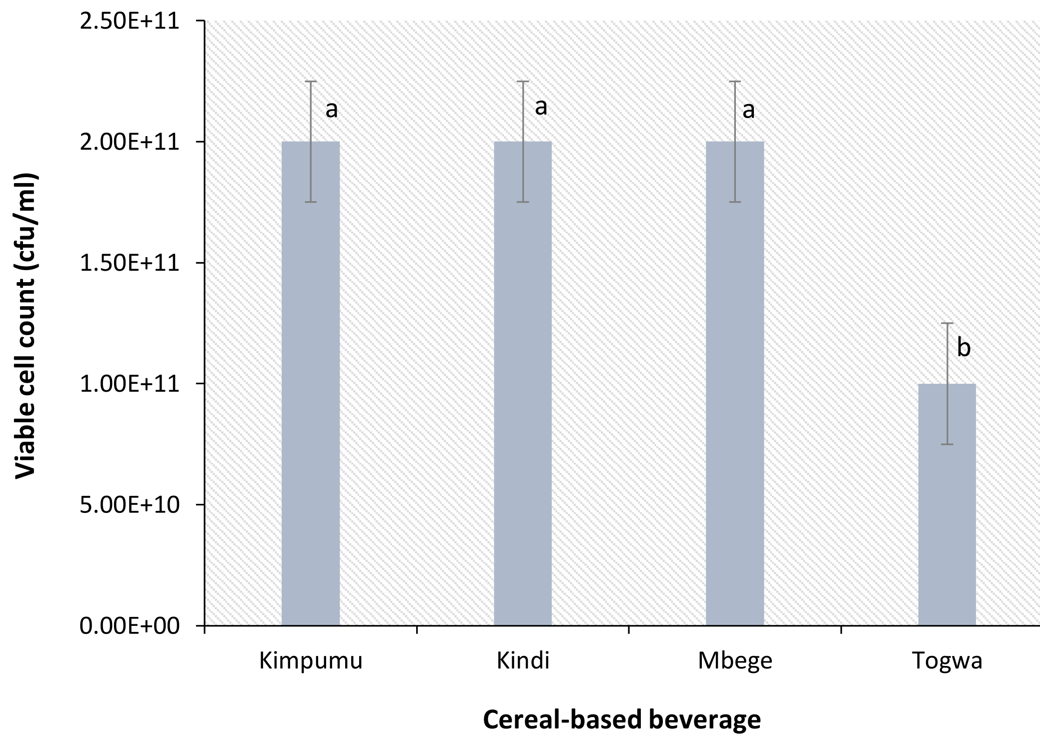

Figure 18: Viable cell counts of the probiotic cereal-based beverages stored at 4 °C for 28 days, error bars represent Mean ±SD, n = 40. The bars stand for viable cell counts

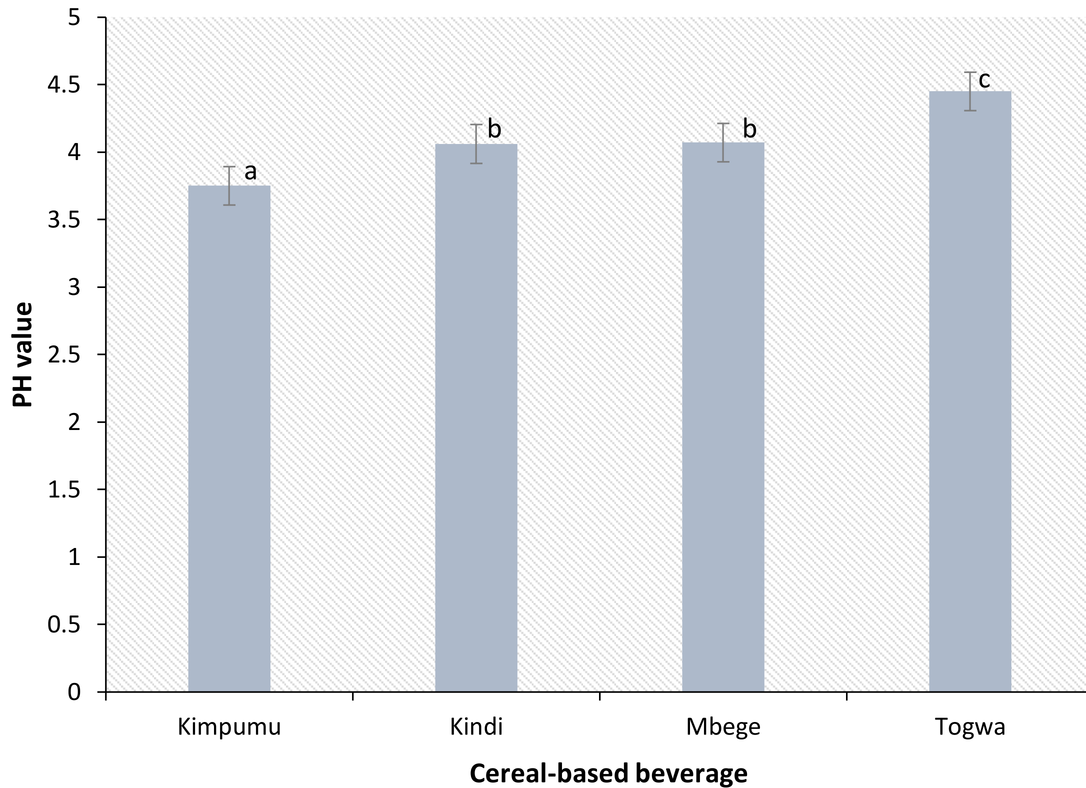

Figure 19: The pH values of the probiotic cereal-based beverages preserved at 4 °C for 28 days, error bars represent Mean ±SD, n = 40. The bars stand for pH values

Furthermore, research findings have indicated that the pH values of the probiotic cereal beverages were varying irregularly with time swinging between pH 3.5 – 4.83 (Fig. 20). The corresponding viable cell counts were above the threshold value of 10^6 cfu/mL for probiotic products (Fig. 21).

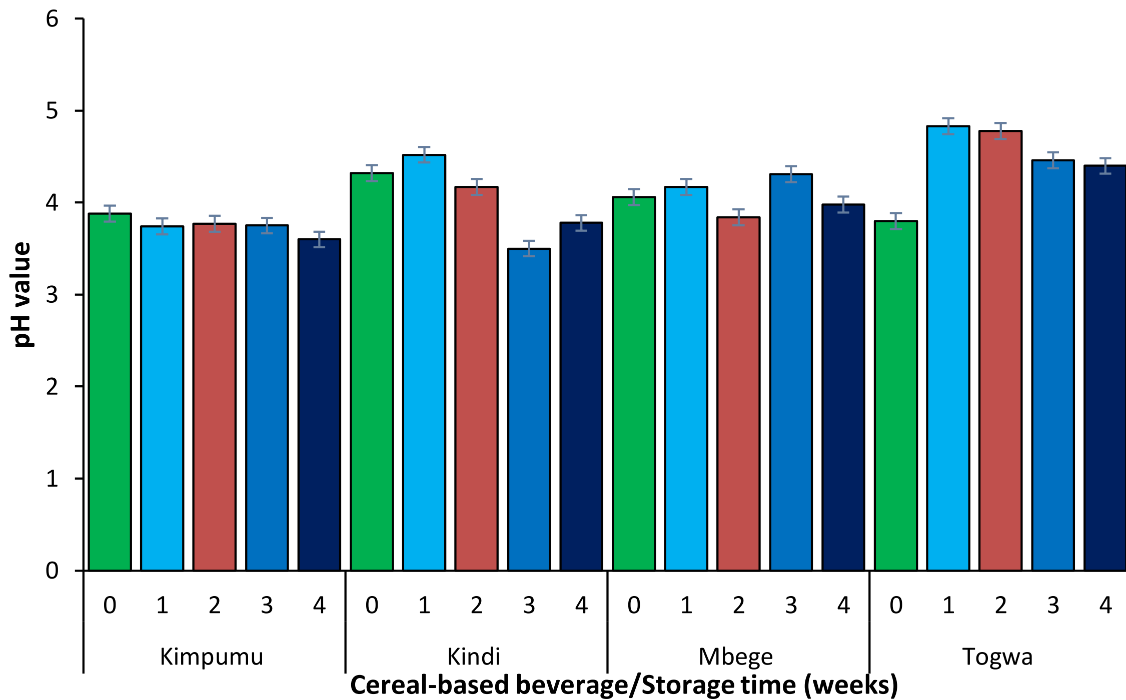

Figure 20: Weekly mean pH values for probiotic cereal-based beverages stored at refrigeration temperature (4 °C), error bars represent Mean ±SD, n = 40. The colored bars stand for pH values

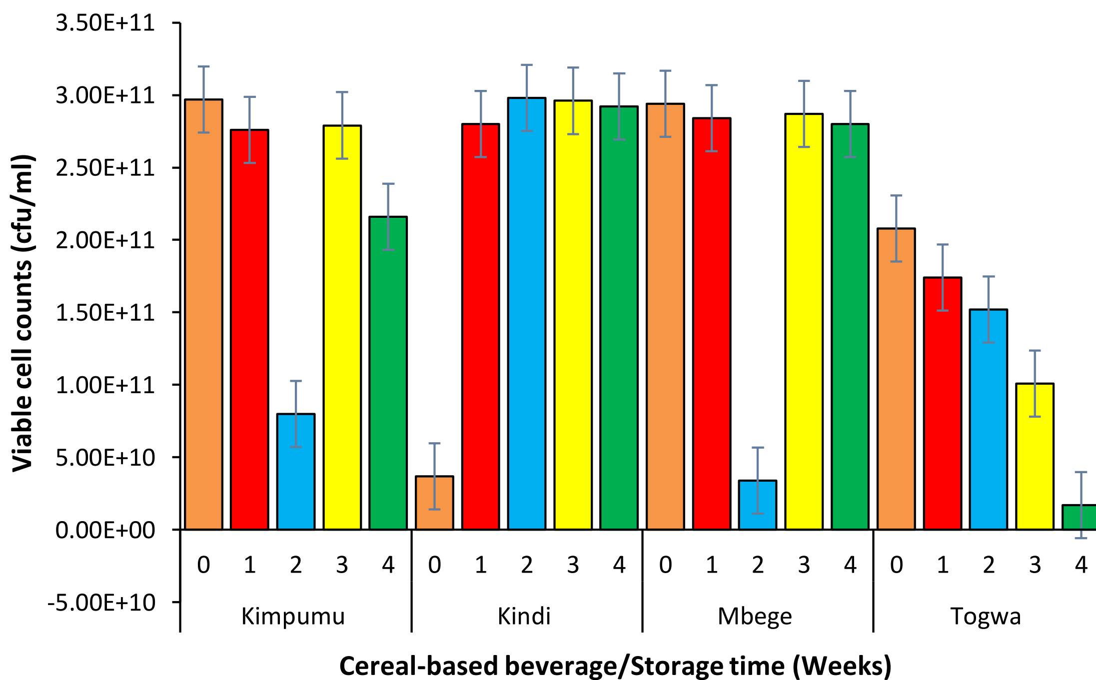

Figure 21: Weekly mean viable cell counts for probiotic cereal-based beverages stored at refrigeration temperature (4 °C), error bars represent Mean ±SD, n = 40. The colored bars stand for viable cell counts

The above results carry the message that when the *Kimpumu, Kindi, Mbege* and *Togwa* were prepared using pure mono-cultures of *L. plantarum*, *P. pentosaceus*, *L. plantarum*, and *L.*

brevis, respectively under 24 h controlled fermentation at 37 °C; the end products when stored at refrigeration temperature were still probiotic cereal-based beverages with pH range of 3.75 – 4.45, cell viability of 2.0 x 10^{11} cfu/mL and a shelf-life of 28 days (four weeks). The pH values of the probiotic cereal beverages were fluctuating for each week of the four weeks of storage at 4 °C reaching a minimum of pH 3.5 in 3 weeks (*Kindi*) and a maximum pH 4.83 (*Togwa*) in the first week (Fig. 20). However, there was a steady decrease of pH for *Kimpumu*. The corresponding viable cell counts were all above the minimum therapeutic value of 10^6 cfu/mL for probiotic products (Fig. 21). At the start of the cereal-based beverage storage (week 0); the viable cell counts for *Kimpumu*, *Mbege* and *Togwa* had already reached the maximum of approximately 3.00 x 10^{11} cfu/mL (*Kimpumu* and *Mbege*) and 2.08 x 10^{11} cfu/mL (*Togwa*) compared to 3.70 x 10^{10} cfu/mL for *Kindi* respectively (Fig. 21). The pH of the cereal-based beverages was then in the range of 3.80 – 4.32 (Fig. 20). This shows that the required number of microbial cells can be achieved through fast controlled fermentation (Freire *et al*., 2017; Mukisa *et al*., 2017). Starting with the first week to the last, there was a decrease in the viable cell counts and corresponding pH values for *Togwa* because the proliferation of *L. brevis* was being hindered by the gradual decrease in pH (Fig. 20). On the other hand, the viable cell counts for *Kindi* was increasing from the first week to the fourth week reaching about 2.9 x 10^{11} cfu/mL (Fig. 21) within a pH range of 3.5 – 4.52 (Fig. 20). However, the viable cell counts for *Kimpumu* and *Mbege* decreased noticeably in the second week due to possibly microbial interference with some metabolic products and slight pH changes but increased again in the third week after acclimatization with the media environment (Gupta & Bajaj, 2017) and reached the final viable cell counts of 2.16 x 10^{11} cfu/mL and 2.80 x 10^{11} cfu/mL, respectively. The viable cell counts of *Togwa* was decreasing with time and this is likely due to the perpetual decrease in pH in the beverage which interfered with the growth of the microbe. Even though, the final viable cell count was 1.7 x 10^{10} cfu/mL above the minimum 10^6 cfu/mL for probiotic foods. Thus, within the 4 weeks of storage it was possible to have large populations of LAB in the beverages high above the minimum 10^6 cfu/mL for probiotic foods making them potentially probiotic in nature. These locally available beverages when prepared under the given conditions can be used to provide the people in Tanzania and elsewhere the much-needed health benefits that are imparted by the probiotic LAB they contain. Such benefits include prevention and cure of diseases such as CVD, diabetes, high blood pressure (BP), infantile diarrhea, and lactose intolerance (Angelov *et al*., 2018).

Similar results were obtained by Di Stefano *et al*. (2017) when he stored a probiotic formulation (10% millet in water, 5% sucrose) for 56 days at 4 °C and found that the product had a viable cell count of up to more than 8log cfu/mL at the end of the storage time. It has also been reported that probiotic fermented oat flour beverage (PFOF) containing *L. plantarum* was found to remain stable (with high viability) even after 4 weeks of refrigerated storage at 4 °C (Gupta & Bajaj, 2017). The stability of the product was attributed to low metabolic activity of the bacterium at refrigerated storage leading to decreased production of lactic acid and hence slight changes in pH of the substrate. A study carried out by Hassan *et al*. (2012) on developing probiotic cereal-based beverage (millet milk fermented with ABT-2 starter culture, sesame and pumpkin seed milks added after fermentation) that was then stored at 4 °C revealed that the product remained stable with pH above 4.0 and probiotic viable count of above 8logcfu/mL for 15 days. The preceding results are comparable to the results in this study and further confirm the possibility of maintaining the viability of probiotic LAB high above 10^6 cfu/mL for 28 days under refrigeration condition.

The findings narrated above are novel in Tanzania and are going to revolutionize the small-scale and medium-scale entrepreneurs; particularly Tanzanian low-income household women who engage in the business of preparing and selling locally made cereal-based beverages. They must improve the way they produce and preserve such products in line with outcomes of this research when validated so as to impart health benefits to beverage consumers, increase their incomes and consequently improve their livelihoods as well as that of their families.

4.11 Safety of the probiotic cereal beverages

Results indicated that there was no growth on Brilliant Green Agar (BGA) which showed absence of *Salmonella spp*. in the probiotic cereal-based beverages. Likewise, there was no growth on Salmonella-Shigella agar which otherwise could indicate the presence of *Shigella spp.* in the probiotic cereal beverages. Furthermore, no visible growth (purple-pink colonies) was observed on VRBGA confirming the absence of *E. coli*. Consequently, these findings provide an empirical fact that the storage of the four cereal-based probiotic beverages at 25 °C for 5 days and at 4 °C for 28 days results to fermented cereal beverages which are free from contaminating agents such as *Salmonella spp*., *Shigella spp.*, and *E. coli* provided that they are stored in hygienically sound containers free from *Enterobacteriaceae*. The safety of the probiotic beverages is ensured by low pH in the range 3.4 to 4.83 (Figure 17 and 20) in the beverages which kills all non-acidophilic bacteria leaving the acidophilic LAB. Various

metabolites such as organic acids, aldehydes, ketones, acetates and several others produced by the LAB during fermentation of the beverages also help in increasing the inhibitory properties of such products (Aka *et al*., 2014; Oyedeji *et al*., 2013). Similar results on safety of functional cereal beverages were observed by Freire *et al*. (2017) through quick and satisfactory acidification of a cereal-based beverage as well as production of various organic acids that improved the safety and impeded the growth of pathogens. In another study, Gupta and Bajaj (2017) reported that there was no *Enterobacteriaceae* contamination in probiotic fermented oat flour beverages stored at 25 °C and 4 °C even after 4 weeks of storage.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This study indicate that the two major genera of microbes involved in the fermentation of the *Kindi*, *Kimpumu*, *Togwa* and *Mbege* were *Lactobacillus* and *Candida*. *Kimpumu* was proliferated by *L. plantarum*, *C. zeylanoides, C. albicans, and C. tropicalis*. *Kindi* was dominated by *P. pentosaceus, C. gattii, C. tropicalis and R. minuta*. *Mbege* was proliferated by *L. plantarum, C. ciferrii* and *C. dubliniensis* whereas *Togwa* was dominated by *L. pentosus, L. brevis, L. plantarum* and *C. tropicalis.*

Single and mixed cereal-based probiotic beverages can be obtained through controlled fermentations at 37 °C for 24 h by means of carefully selected monocultures of probiotic LAB. Assessment of probiotism of the four cereal-based beverages prepared under controlled fermentation conditions has indicated that the resultant products had probiotic characteristics with viable cell counts of 1.0×10^{11} cfu/mL and pH of 4.77.

Sensory evaluation has shown that the four-probiotic cereal-based beverages have good and promising quality attributes in terms of appearance, color, taste, aroma, texture and overall acceptability. Cereal-based probiotic *Togwa* was the most preferred of the four prepared cereal-based probiotic beverages in this study due to its better organoleptic quality attributes.

The study has further revealed that the optimization of the fermentation processes of the cereal-based beverages produces probiotic products that maintain their stability at ambient temperature (25 °C) for 5 days with pH of 3.83 and viable cell counts of 2.0×10^{11} cfu/mL while at 4 °C their stability is 28 days with pH of 4.08 and viable cell counts of 2.0×10^{11} cfu/mL. Decreasing pH of the cereal-based probiotic beverages during both ambient and refrigeration storage temperatures ensured safety of the beverages.

The novelty of this study is the optimization of cereal fermentation processes of the locally made cereal-based beverages to produce improved cereal-based probiotic beverages. Overall, this study has advanced the knowledge that fermented cereal substrates are promising nutrient-rich media for probiotic delivery to human being by sustaining the growth of large populations of LAB.

5.2 Recommendations

(i) There is a need to do further studies on production of probiotic cereal-based beverages in Tanzania in order to validate the results of this study and establish the basis upon which producers of fermented cereal-based beverages will upscale the validated results. Commercialization of the products is of important consideration for provision of health-giving beverages to the majority of Tanzanians and at the same time boost the incomes of the beverage producers.

(ii) It is recommended that probiotics; particularly LAB isolated and identified in this study can be used as starter cultures for optimization and upscaling production of the studied local cereal-based beverages.

(iii) Use of Analytical Profile Index (API) systems e.g., API 50 CHL for identification of microbes after isolation and characterization is very useful but sometimes biochemical tests may give similar identity to microbes which show similar characteristics though they might be from different species. Consequently, molecular techniques are essential part in microbial identification.

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APPENDICES

Appendix 1: Sensory evaluation form

Consumer acceptance/preference test

Instructions:

a) Fill this evaluation form correctly and follow the given instructions
b) You are given four (4) coded samples of health promoting cereal beverages for evaluation
c) Please, rinse your mouth after each sample tasting using potable water provided
d) Don't seek another person help

Names: ………………………………………… **Date…**…………………**Time**…………

Please answer questions 1 and 2 by putting a tick (√) in the boxes provided.

1. Your gender ☐ Male ☐ Female

2. Age (years): ☐ 15-25 ☐ 26-35 ☐ 36-45 ☐ 46-60 ☐ > 60

3. Evaluate each of the four (4) coded product samples from left to right. Using the 7-point hedonic scale (elaborated below) indicate how much you like or dislike each sample by evaluating the appropriate sample attributes. (Put a score in the box against each attribute).

7-point hedonic scale: 1-Like extremely, 2-Like moderately, 3-Like slightly, 4-Nether like nor dislike, 5-Dislike slightly, 6-Dislike moderately, 7-Dislike extremely.

Attributes	**Coded samples**			
	119	**346**	**269**	**959**
Appearance				
Color				
Taste				
Odor/aroma				
Texture				
Overall acceptability				

Write your comments below by using the sample codes
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4. Which coded sample do you prefer most (tick √ one box)?
☐ 119 ☐ 346 ☐ 269 ☐ 959

5. How do you evaluate the **sweetness** of the given product samples? Tick one box

	Too low	A little low	Just right	A little high	Too high
119	☐	☐	☐	☐	☐
346	☐	☐	☐	☐	☐
269	☐	☐	☐	☐	☐
959	☐	☐	☐	☐	☐

6. How do you evaluate the **Acidity** of the given product samples? Tick one box

	Too low	A little low	Just right	A little high	Too high
119	☐	☐	☐	☐	☐
346	☐	☐	☐	☐	☐
269	☐	☐	☐	☐	☐
959	☐	☐	☐	☐	☐

7. How do you evaluate the **taste/flavor** of the given product samples? Tick one box

	Too low	A little low	Just right	A little high	Too high
119					
346					
269					
959					

8. How do you evaluate the **aroma/odor** of the given product samples? Tick one box

	Too low	A little low	Just right	A little high	Too high
119					
346					
269					
959					

9. How do you evaluate the **color** of the given product samples? Tick one box

	Too low	A little low	Just right	A little high	Too high
119					
346					

269	☐	☐	☐	☐	☐
959	☐	☐	☐	☐	☐

10. How do you evaluate the **texture** of the given product samples? Tick one box

	Too low	A little low	Just right	A little high	Too high
119	☐	☐	☐	☐	☐
346	☐	☐	☐	☐	☐
269	☐	☐	☐	☐	☐
959	☐	☐	☐	☐	☐

11. Would you think of purchasing these products? Tick one box for each sample.

	YES	NO
119	☐	☐
346	☐	☐
269	☐	☐
959	☐	☐

12. Provide any other comments regarding the evaluated products making use of the sample codes

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Thank you for your cooperation

RESEARCH OUTPUTS

(i) Journal papers

Setta, M. C., Mbega, E. R., & Matemu, A. O. (2021). Screening local cereal-based beverages in Tanzania for yeast contaminants. *International Journal of Biosciences*, *18*(2), 194-201. http://dx.doi.org/10.12692/ijb/18.2.194-201

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Submitted

Probiotic cereal-based beverages produced from improved recipes of local cereal-based drinks in Tanzania

(ii) Poster Presentation

Optimization of fermentation processes of local cereal-based beverages to produce improved cereal-based probiotic beverages