MICROBIOTA OF FERMENTING MILLET IN *HAUSA KOKO* PRODUCTION: THEIR DIVERSITY, FERMENTATIVE CHARACTERISTICS AND POTENTIAL FOR STARTER CULTURE DEVELOPMENT

BY
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A THESIS SUBMITTED TO UNIVERSITY OF GHANA, LEGON, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY (PHD) FOOD SCIENCE DEGREE

DEPARTMENT OF NUTRITION AND FOOD SCIENCE
FACULTY OF SCIENCE
UNIVERSITY OF GHANA
LEGON

DECEMBER, 2021

Dedication

I dedicate this work to the Almighty God and give Him all the glory for successfully seeing me through this research work. I am forever grateful. Also, to my sweet and loving husband, Isaac Atter, and son, Ivan Atter, for their understanding, motivation, sacrifices and prayers. Not forgetting my supportive parents, Togbe Odoom Kumato III and Madam Judith Sah. A special dedication to my late son, Emmanuel Atter, you are loved always.

Declaration

I hereby declare that, except for references to the work of others that have been duly cited, this work is the result of my own original research under the supervision of my main supervisor, Prof. Kwaku Tano-Debrah and co-supervisors, Prof. Wisdom Amoa-Awua, Dr. Angela Parry-Hanson Kunadu and Prof. Arjan Narbad, and that this thesis either in whole or part has not been presented for another degree elsewhere.

……. 16th December 2021

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Acknowledgements

This study was financially supported by UK Biotechnology and Biological Sciences Research Council (BBSRC) via a Global Challenge Research Fund Data and Resources award and Institute Strategic Programmes for Food Innovation and Health (BB/R012512/1, and its constituent projects which I am very grateful to. I am extending my special thanks to Prof. Arjan Narbad, my co-supervisor and the group leader of the Gut Microbes and Health Institute Strategic Programme, Quadram Institute Bioscience (QIB), Norwich Research Park, Norwich, United Kingdom. Thank you for accepting me and providing every necessary support to carry out this research work successfully. To Dr. Maria Diaz, I sincerely appreciate all your tolerance and invaluable contribution to the success of this study. Your readiness to provide every needed assistance and your pleasant nature made it really gratifying and a pleasure working with you Dr. Melinda Mayer. To Drs Lizbeth Sayavedra, Lee Kellingray, Gang Wang, Ebenezer Foster-Nyarko, Steve James, Andrea Telatin, Ian Colquhoun and all other QIB staff, visiting students and the entire research group members that I cannot mention here, a big thank you for your support in diverse ways.

I wish to express my sincere appreciation to my lead supervisor Prof. Kwaku Tano-Debrah who guided me throughout this study and had to embark on a retreat just to review this work thoroughly. To Prof. Wisdom Amoa-Awua, you are not just a co-supervisor but my earthly 'guardian angel' a father, friend, mentor, life coach who has supported me throughout my career. Words cannot express how grateful I am. Your constructive suggestions, review and scrutiny is well appreciated Dr. Angela Parry-Hanson Kunadu, my co-supervisor.

My special thanks also goes to Prof. Matilda Steiner-Asiedu for all the encouragement and support in diverse ways. Prof. Dennis Nielsen (University of Copenhagen, Denmark) you are well

appreciated. I would like to say thank you for your support and words of encouragement Profs. Mary Obodai and Charles Tortoe, immediate past and current Directors of CSIR-Food Research Institute, staff and management. The Head of Department of Nutrition and Food Science, Dr. Frederick Vuvor for his immense support as well as other senior members and staff of the department.

I also recognize the selfless assistance from my colleagues and friends Messrs. Kojo Odei Obiri, Afotey Alemawor, Chris Galley, Evans Agbemafle, Frank Peget, Papa Toah Akonor, Raphael Kavi, Vincent Kyei-Barfoe, Hillary Ketemefe, Theophilus Annan, Stephen Nketia, Felix Kwashie Madilo, Edward Archer, Crossby Osei-Tutu, George Anyebuno, Nelson Ameh, Derick Salla, Eric Ofori; Mmes Anthonia Andoh-Odoom, Serwa Buckman, Matilda Dzomeku and Dora Ofori Appiah; Drs Margaret Owusu, Charlotte Oduro-Yeboah, Dzifa Dey, Hayford Ofori and Ethel Blessie.

I could not have asked for anything more Rev. and Mrs. Kingsley Tetteh, Rev. and Mrs. Jeremiah Thirdson, Rev. and Mrs. Arnold Nyadi, Rev. and Mrs. Ernest Agyei, Rev. and Mrs. Benjamin Tettey, Pastor and Mrs. Boateng Sarpong, Princess Sunu, Theresa Amoah, Ruby Tawiah Ntiri, Josephine Gomashi as well as Marcellina, Peter, Aaron, Paa Kodzo, your prayers and support are well appreciated.

Abstract

Hausa koko is a traditional free -flowing spicy fermented pearl millet porridge produced mostly at the household level by women and sold as street food in Ghana. The fermentation is spontaneously done, not controlled, and the final product prone to contamination with potential foodborne pathogens. To standardize and control the fermentation process to achieve better product quality and safety for large-scale production, the use of starter culture containing beneficial fermenting microbes such as lactic acid bacteria (LAB) and yeast was considered in this study. Samples at different processing stages were obtained from twelve (12) different commercial processors located in six regions of Ghana. Their bacterial community were determined using the V4 variable region of the 16S rRNA gene and their metabolite profiled using ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy. Out of the 12 commercial processors, samples from five (5) were enumerated for lactic acid bacteria (LAB) and yeast. The LAB isolates were fingerprinted using (GTG)5 based rep-PCR before whole genome sequenced, while 28S rRNA genes were Sanger sequenced for yeast isolates for genetic characterisation and identification. The identified isolates were then screened for some technological and probiotic characteristics in-vitro, beneficial isolates were used to develop starter culture whose performance was evaluated in-situ and tested for consumer acceptability. Results revealed the most comprehensive bacterial community with over four hundred (400) different Gram-positive and Gram-negative organisms and profiled thirty-three (33) metabolites. The LAB isolates were made up of both homo and hetero fermentative organisms. They included *Limosilactobacillus pontis* (31.11 %), *Pediococcus acidilactici* and *Limosilactobacillus fermentum* (16.67 % each), *Pediococcus pentosaceus* (11.11 %), *Limosilactobacillus reuteri* (10 %), *Weissella confusa* (6.67 %), *Schleiferilactobacillus harbinensis* (3.33 %), *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei* (2.22 %

each). *L. pontis, L. fermentum, P. pentosaceus* and *L. reuteri* occurred at all the stages of *Hausa koko* production. *Saccharomyces cf. cerevisiae/paradoxus* (41.4 %), *Saccharomyces cerevisiae* (31.0 %), *Pichia kudriavzevii* (13.8 %), *Clavispora lusitaniae* (8.6 %) and *Candida tropicalis* (5.2 %) were the yeast identified. A total of 27 LAB isolates were predicted to have bacteriocin producing genes and genes related to nutritive and enzyme production. Subsequently, these isolates were selected for further testing. The majority of the selected LAB and yeast isolates exhibited good technological and potential probiotic characteristics *in vitro*. The LAB showed good rates of acidification, strong inhibitory activity against some foodborne indicator organisms, amylase production (66.7 %), and low production of exopolysaccharides (EPS) (33.3 %). They also exhibited good tolerance and survival in acid conditions at pH (2.5 - 6.0) and at pH 7, tolerance and survival against bile (0.3 - 1.0 %). Similar good probiotic characteristic was obtained from the yeast isolates including tolerance to low-neutral pH (2.0, 3.0, 5.5 and 7.0), bile (0.3 - 1.0 %), high temperatures (25 °C and 37 °C) and salt concentrations (4 and 6 %). LAB isolates *L. reuteri* LDOD-Sud, *L. pontis* LTAD-12g and *L. fermentum* LMAN-Sdb, and yeast isolates, *S. cerevisiae* YSUN-Sud and *P. kudriavzevii* YTAD-12j selected for further studies in the development of a starter culture or inoculum enrichment during millet fermentation in different combinations produced acceptable results. Reduction or total inhibition of aflatoxins B1, B2 and G2 infected millet slurries were recorded when fermented with the different combinations. The most preferred starter culture combination was *Limosilactobacillus reuteri* LDOD-Sud (R) + *Limosilactobacillus fermentum* LMAN-Sdb (F) + *Saccharomyces cerevisiae* YSUN-Sud (C) referred to as RFC *Hausa koko* Starter Culture (RFCH). Although not under a strictly controlled fermentation set up, RFCH starter culture demonstrated desirable traits including quality and safety improvement, reduced fermentation time from the normal 48 - 72 h to only 12 h during semi-industrial scale fermentation.

The study presented the most comprehensive bacterial and metabolites profile, the diversity, technological and probiotic potential of microorganisms associated with *Hausa koko* processing and the development of a starter culture. Also, it has shown the possibility of using starter culture by commercial processors at semi-industrial scale to standardize the production process for improved quality and safety.

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CHAPTER ONE

1.0 Introduction

1.1 *Hausa koko* production in Ghana

Fermentation of cereals both at household and semi-industrial scale is a very popular practice in Ghana. Pearl millet (*Pennisetum glaucum*) is one such cereal used extensively. It is a versatile cereal crop, which provides food, fodder and fuel; and it is produced on more than 27 million hectares of land worldwide (Jalaja et al., 2016). It is an important crop in the semi-arid regions in the world and due to its nutritional content (Jaybhaye et al., 2014; Yadav et al., 2014; Sade, 2009; Lestienne et al., 2007; Malleshi & Klopfenstein, 1998), it contributes to the dietary need of the people. Either whole or dehulled, pearl millet is mostly processed and spontaneously fermented into a dough that is subsequently used in the preparation of numerous indigenous dishes in liquid or semi-solid states. These foods are consumed daily as breakfast, lunch, snacks and complementary foods. The main fermented foods produced from pearl millet in Ghana are *fura*, *maasa*, *brukina* and a very popular porridge known as *Hausa koko* (Amoo-Gyasi, 2013; Owusu-Kwarteng & Akabanda, 2013; Owusu-Kwarteng et al., 2010; Lei & Jakobsen, 2004).

Koko is the general term for thin porridges made from any fermented cereal in Ghana. Thin porridges made from fermented millet is called *Hausa koko.* The word *'Hausa'* is added to the *'koko'* because it is more associated with Hausa-speaking people. *Hausa koko* is usually prepared at household level by women. It is consumed by children and adults of all social classes mostly at breakfast and recognized as a national street food. *Hausa koko* is mostly served hot with sugar to taste and sometimes milk, and consumed with accompaniments such as peanuts, bread, *koose*

(fried cowpea paste), *maasa* (fried millet, rice or maize paste) or doughnuts commonly called *bofrot* (Haleegoah et al., 2016; 2015). *Hausa koko* was previously studied by Lei & Jakobsen (2004). The lactic acid bacteria (LAB) isolates from the spontaneously fermented porridge samples from five processing sites in Northern Ghana were characterised by sequencing of their 16S Ribosomal ribonucleic acid (rRNA) gene. They reported the presence of *W. confusa, L. fermentum, L. salivarius* and *Pediococcus spp.* Other species observed in *koko* sour water were *L. salivarius, P. pentosaceus, P. acidilactici* and *L. paraplantarum.*

Lei & Jakobsen (2004), described a process of *Hausa koko,* production (Figure 1(A)), which depicts the fermentation characteristics of the product. Figure 1(B) also shows the process flow of a variant preparation method. Differences between the previous process flow (Figure 1(A)), and the current process flow (Figure 1(B) include longer grain steeping and longer slurry fermentation times. The variations could likely influence the microbial diversity of the final product.

In recent years, there has been a rise in semi-industrial production of some indigenous foods including *Hausa koko* where it is processed and packaged into disposable take-away cups. This form is more expedient since the package sometimes comes enclosed with sugar, sachet of milk, tissue paper, disposable spoon, bread and other accompaniments. There is also the *Hausa koko* powder (fermented millet powder or packaged oven dried, milled fermented millet mixed with spices, for self-preparation at home). These have been made possible through several attempts to mechanize and improve the quality of traditional food processing in Ghana.

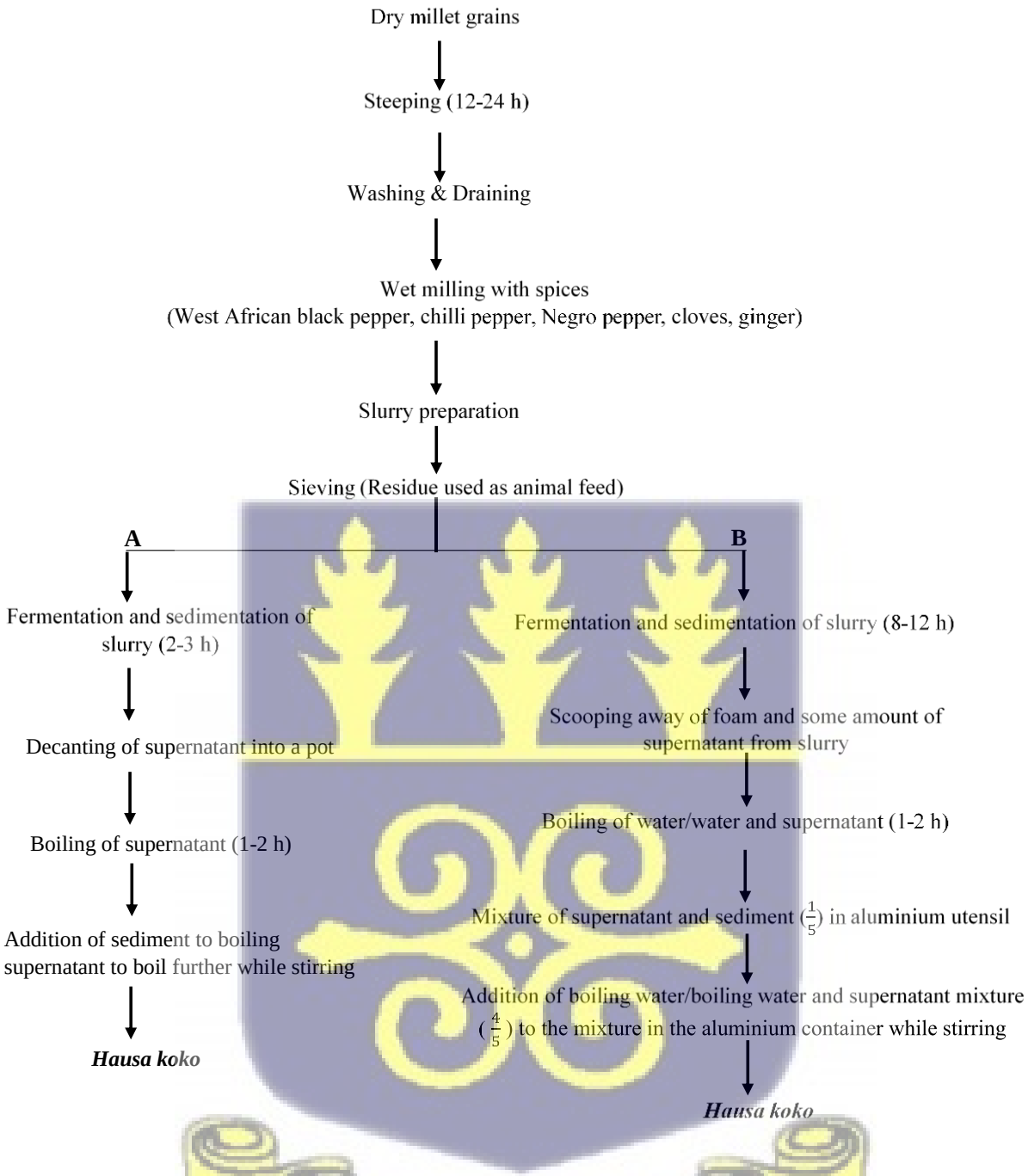


Figure 1: Flow diagram of modified *Hausa koko* production process, (A: step described by Lei & Jakobsen (2004), B; step described in current work)

1.2 Rationale

The fermentation steps in the *Hausa koko* processes still occur spontaneously and are caused by the native microflora contaminating the grains and the dough or slurry; which may include both beneficial and potentially harmful microorganisms. Very little control of the steps are achieved with the fermentation time and probably the cleaning and milling operations that may influence the microflora and may be the dough or slurry preparation. The inadequate control of the fermentation could result in products of varying quality and safety, and thus constitutes a limitation in the achievement of the semi-industrialisation efforts.

Attempts are being made to adequately control indigenous fermentation processes in Ghana, including starter culture application (Halm et al.,1996; Lei & Jakobsen, 2004). A guidance in the design of starter cultures requires an in-depth study into the microbial diversity using culture-independent high throughput sequencing technology and determine the metabolites profile. The use of a starter culture containing lactic acid bacteria (LAB) and yeast with defined beneficial traits for the fermentation process is expected to add greatly to the product safety and quality. There are yet, no starter cultures for the production of *Hausa koko*; and there is the need to develop them. To develop a starter culture, however requires an in-depth appreciation of the microbial diversity of the *Hausa koko* processes across the different geographical locations. There is the need to identify the predominant fermenting microflora, in this regard, lactic acid bacteria (LAB) and yeasts, of desirable technological properties for starter culture development. The study purpose was to identify and characterise the LAB and yeasts associated with the processing of millet into *Hausa koko* in Ghana, using phenotypic and genomic methods such as Sanger sequencing, 16S rRNA gene amplicon sequencing and whole genome sequencing (WGS). The Genomic methods have high sensitivity, show high discrepancy and high accuracy; and could provide a greater depth

of information about the fermenting microorganisms. In addition to the high sensitivity of the genomic methods, the use of bioinformatics tools was to allow for high level data analysis that could be used for pre-screening and selection of LAB cultures before thorough microbial characterisation. Combination of sequencing data and bioinformatic tools alongside phenotypic methods provides a powerful approach to define microbial clusters which can be used to predict relative contributions to food fermentation.

1.3 Main objective

The aim of the study was to characterise the microbiota involved in *Hausa koko* production and investigate the improvement of the quality and safety of the product using beneficial starter cultures indigenous to *Hausa koko*.

1.4 Specific objectives

The specific objectives of the study were:

1. To determine the bacterial community at different stages of *Hausa koko* production using culture-independent 16S based metagenomics sequencing and quantifying their metabolites.
2. To conduct genetic characterisation of the dominant lactic acid bacteria (LAB) and yeasts using Illumina WGS and 28S rRNA region respectively.
3. To determine the technological and probiotic properties of the selected beneficial LAB and yeast from pearl millet fermentation during *Hausa koko* production.
4. To develop a starter culture using probiotic cultures of LAB and yeasts isolated from traditional spontaneous fermentation of pearl millet during *Hausa koko* production.

5. To determine the performance of the starter culture developed for the fermentation of pearl millet.

1.5 Outline of thesis

The outline of the thesis is as follows:

I. Chapter 1 - Introduction

II. Chapter 2 - Literature review

III. Chapter 3 - Bacterial diversity and metabolites profiling during *Hausa koko* production

IV. Chapter 4 - Lactic acid bacteria and yeasts associated with the traditional fermentation of millet in *Hausa koko* production

V. Chapter 5 - Technological and probiotic properties of LAB and yeast from *Hausa koko*, a millet based porridge

VI. Chapter 6 - Starter culture development

VII. Chapter 7 - Pilot and Semi-Industrial Scale testing of the use of Starter Cultures in *Hausa koko* Production

VIII. Chapter 8 - General discussion, conclusions and recommendations

1.6 Ethical Consideration

Ethical consideration was sought from Ethics Committee for Basic and Applied Sciences (ECBAS), University of Ghana for the study. Approval was granted both for the initial application

and subsequent amendment with certified protocol Number ECBAS 014/19-20 for the study. The concent of participants were sought.

CHAPTER TWO

2.0 Literature review

2.1 Fermented foods

Fermentation is the process that transforms food substrates into new products through the action of microorganisms and the biochemical changes result in the modification of the substrates and production of essential compounds (Singh et al., 2015). Even though any food that has undergone any form of fermentation process can simply be described as fermented food, there is a variety of descriptions in literature. According to Campbell-Platt (1987), fermented foods are foods that have been subjected to the action of microorganisms and enzymes resulting in the bioconversion of raw materials into foods that bear unique characteristics that are different from the original raw material. Similarly, Blandino et al., (2003) described fermented foods and beverages as those that have been subjected to the effect of microorganisms or enzymes to produce desirable biochemical changes.

2.2 Classification of fermented foods

The physical, biological or chemical properties of fermented foods may be used in their classification. There may be over 5000 different common and uncommon fermented foods and beverages in the world with diverse classifications. These can be classified into nine (9) main categories based on the raw material or substrate used (Tamang, 2010; Steinkraus, 1997). They include cereals, legumes, meat products, dried also smoked fish products, alcoholic beverages, miscellaneous fermented products and others.

Based on the substrates or raw materials used in the processing of the fermented foods, they can be grouped, according to Achi (2005), as follows:

- Fermented cereals e.g. *ogi*
- Fermented starchy foods e.g. *gari*
- Fermented legumes and oilseeds e.g. *iru, dawadawa, ogiri, okpiye*
- Fermented animal proteins e.g. *nono*, yoghurt
- Alcoholic beverages e.g. *burukutu, pito, obiolor*

Odunfa (1985) also classified African fermented foods under the following four commodity groups:

- Fermented vegetable proteins e.g. *iru, ogiri*
- Fermented animal proteins e.g. *nono, momoni*
- Fermented non-alcoholic starchy foods e.g. *ogi, gari*
- Alcoholic beverages e.g. *burukutu*, kaffir beer, palm wine

Another classification of fermented foods categorised them into 8 groups. Even though there are no clear distinctions in some of these classifications, they still have been found very useful and widely referred to (Steinkraus, 2002; 1996; 1983). They include:

1. Fermentations producing textured vegetable protein meat substitutes found in legume and or cereal mixtures such as *ontjom.*

2. High savory meat-flavored/amino acid/peptide sauce and paste fermentations like the Chinese soy sauce and Indonesian *kecap*,

3. Lactic acid fermentations are associated with products that undergo lactic acid fermentations including cereals and tubers (Ghanaian *kenkey*); milks (yogurts, kefir, cheese etc.); vegetable pickles (cucumber pickles, sauerkraut, olives, Thai *pak-sian –dong*).

4. Alcoholic fermentations which are mostly associated with the production of beers, wine so on. In Africa, some of these include Kenyan *busaa*, Zambian maize beer; Ghanaian *pito*, Ethiopian *talla*. Others include grape wines and many more.

5. Acetic acid/vinegar fermentations which occurs in products such as palm wine and coconut water vinegar.

6. Alkaline fermentation which is associated with Japanese *natto*, Indian *kenima*, Thai *thua-nao*, African *iru*, *ogiri*, Ivory Coast *soumbara*, Nigerian or Ghanaian *dawadawa*.

7. Yeast associated with the fermentation of leavened breads such as Middle East breads.

8. Flat unleavened breads.

Fermented cereal-based foods alone can also be classified based on the raw cereal material used (Soro-Yao et al., 2014) as:

i. Maize-based foods e.g. *kenkey, mawe, banku, agidi*

ii. Millet-based foods e.g. *ben-saalga, arraw, dagnan, degue*

iii. Sorghum based foods e.g. *kunun-zaki, kome, gowe, ogi* cereal based food

2.3 Cereal fermentation

The cultivation of cereals alone covers over 73 % of the world's harvested area and provides over 60 % of food production in the world, contributing greatly to the dietary needs of people (Charalampopoulos et al., 2002). The main cereal crops produced globally include rice, maize,

wheat, rye, barley, millet and sorghum and are considered important and a good source of carbohydrates, dietary proteins, irons, trace minerals, fiber and vitamins (De Valdez et al., 2010). They are a good substrate for the growth of probiotic microorganisms and have also been described as functional foods because they contain sufficient quantities of biologically active components that are capable of imparting health benefits to the consumer in addition to the nutrients they provide (Achi & Ukwuru, 2015; Charalampopoulos et al., 2002). Contrary views have also been reported whereby they are sometimes considered inferior due to their deficiency in some essential amino acids, proteins and the presence of anti-nutritive compounds like tannins, phytic acid and phenols (Taylor et al., 2010; Blandino et al., 2003). Foods prepared from unfermented cereals have also been described as lacking flavour and aroma (Achi & Ukwuru, 2015).

Cereals in their dried states are metabolically inactive including the enzymes. Their absorption of water when added stimulates the enzymes into action and subsequent growth and proliferation of microorganisms to start the fermentation process (Achi & Ukwuru, 2015). Fermentation has however been used to overcome these limitations and nutritionally, fermented cereals are considered superior due to the functional properties of the key fermenting microorganism involved. They have probiotic properties, produce metabolites that impart health-promoting benefits, antimicrobial properties which improve the food quality and safety, extend shelf life, antioxidant activity and remove toxic and anti-nutritional compounds. The high content of soluble non-starch polysaccharides, essential vitamins, minerals, proteins, sterols and other nutrients are produced as well (Tamang et al., 2016; Achi & Ukwuru, 2015; Jaybhaye et al., 2014; De Valdez et al., 2010). The low levels of organoleptic compounds in unfermented cereals account for their unappealing sensory characteristics including their taste, flavour and aroma. However, fermentation boosts enzymatic activities producing metabolites such as acids, sugars, alcohols,

esters and many others which enhances the palatability of cereals (Tsafrakidou et al., 2020; Peyer et al., 2016). Cereals also have prebiotic constituents such as polysaccharides, dietary fibers, short oligosaccharides and resistant starch which support the thriving of functional microbes during gastric passage (Macfarlane et al., 2006). They are now considered appropriate raw materials for innovations for functional foods containing both probiotics and prebiotics (De Valdez et al., 2010).

2.3.1 Millet

Particular attention is now being paid to the cultivation of lesser-known cereals like millet. This is is because of the ever-growing population of the world, escalating food prices, scarceness of water, change in climate and other socio-economic impacts. These are great dangers to food security and agriculture particularly to people leaving in arid and sub-arid regions of the world such as Africa and Asia. It is therefore very important to explore possibilities of producing, processing and using food sources that are drought resistant such as millet to put an end to hunger and poverty (Saleh et al., 2013).

Millet is mostly cultivated under harsh conditions which most cereals will not survive or produce significant yield. It is therefore an important crop especially in developing countries (Yang et al., 2012; Amadou et al., 2011; FAO, 2008). Due to its ability to withstand drought conditions and insect damage, it is a very important crop during famine seasons (Adekunle et al., 2013). It belongs to the *Poaceae* (formerly known as *Gramineae*) plant family. About ten (10) different varieties exist but pearl millet forms the main one accounting for up to 40 % of the global production (Yang et al., 2012; Amadou et al., 2011; FAO, ICRISAT. 1996).

The largest producing African countries include Nigeria (41 %), Niger (16%), Burkina Faso (7%), Mali (6.4%), Senegal and Sudan (4.8 % each). Pearl millet is the most cultivated in Africa with a

hectarage of 76 %, followed by finger millet (19 %), tef (9 %) and fonio (4 %). Out of the total production, 78 % is used as a staple food, 20 % for drinks and other uses whilst 2 % as feed (Obilana, 2003).

Just like other West African countries, pearl millet (*Pennisetum glaucum*) is what is cultivated in Ghana. There are two varieties of millet in Ghana now, the improved disease-resistant early maturing (70-75 days to harvest) variety called *Naara* with botanical names like *Afribeh-naara, Waap-naara, Akad-kom, Naad-kohblug, Kaanati* which has good grain yield, and the late maturing (Zea), with botanical names as *Salma I, Salma III and Langbense* and which has poor yield. *Manga naara* was the very first variety introduced in the 1970s and cropped extensively with many pests, diseases and drought challenges. The improved *naara* varieties were introduced by the Council for Scientific and Industrial Research (CSIR) - Savanna Agricultural Research Institute to boost pearl millet production in Ghana (Akayeti, 2019; Kanton et al., 2015). The gross production of millet in Ghana is about 157,369 MT with an estimated net consumption of 142,815 MT according to the 2015/2016 food balance sheet, with the Northern regions being the only producers (SRID, 2016). Northern Ghana is situated in Sudan and Guinea Savanna zone and is marked by a rainfall pattern that spans between April/May and September/October with a yearly average ranging from 800-1200 mm (Bennett-Lartey & Oteng-Yeboah, 2008).

Pearl millet is regarded as superior or equal to many other kinds of cereal in terms of its nutrient content (Obilana, 2003). Besides protein, dietary fat, starch, fiber, it is also rich in minerals, antioxidants and essential amino acids (Amadou et al., 2013; Yang et al., 2012; Ragaee et al., 2006; Ali et al., 2003).

In Ghana, millet, either whole or dehulled, is mostly processed traditionally at the household level where they are milled into flour or spontaneously fermented into a dough and subsequently used

in the preparation of indigenous dishes in liquid or semi-solid states in Ghana. They are used as breakfast, complementary foods, refreshing drinks for snacks and staples like *Tuo Zafi*, *zoom-koom*, *fura*, *maasa*, *burkina /brukina* and *Hausa koko*. The processing method of *fura*, *maasa* and *burkina* which are the most common fermented millet foods are highlighted as follows:

2.3.1.1 *Fura*

Fura is a popular semi-solid dumpling common in Ghana. In *fura* processing (Figure 2), the millet is soaked in water for 18 - 28 h. The grains are washed and wet milled after the addition of spices such as ginger, cloves, mint, and pepper. The resulting dough is cooked for about 30 min and after, hand-molded into balls (approximately 10 cm in diameter). Mortar and pestle are used to pound the cooked millet balls after which they are molded again into much smaller balls and coated with maize flour before the sale (Owusu-Kwarteng et al., 2010).

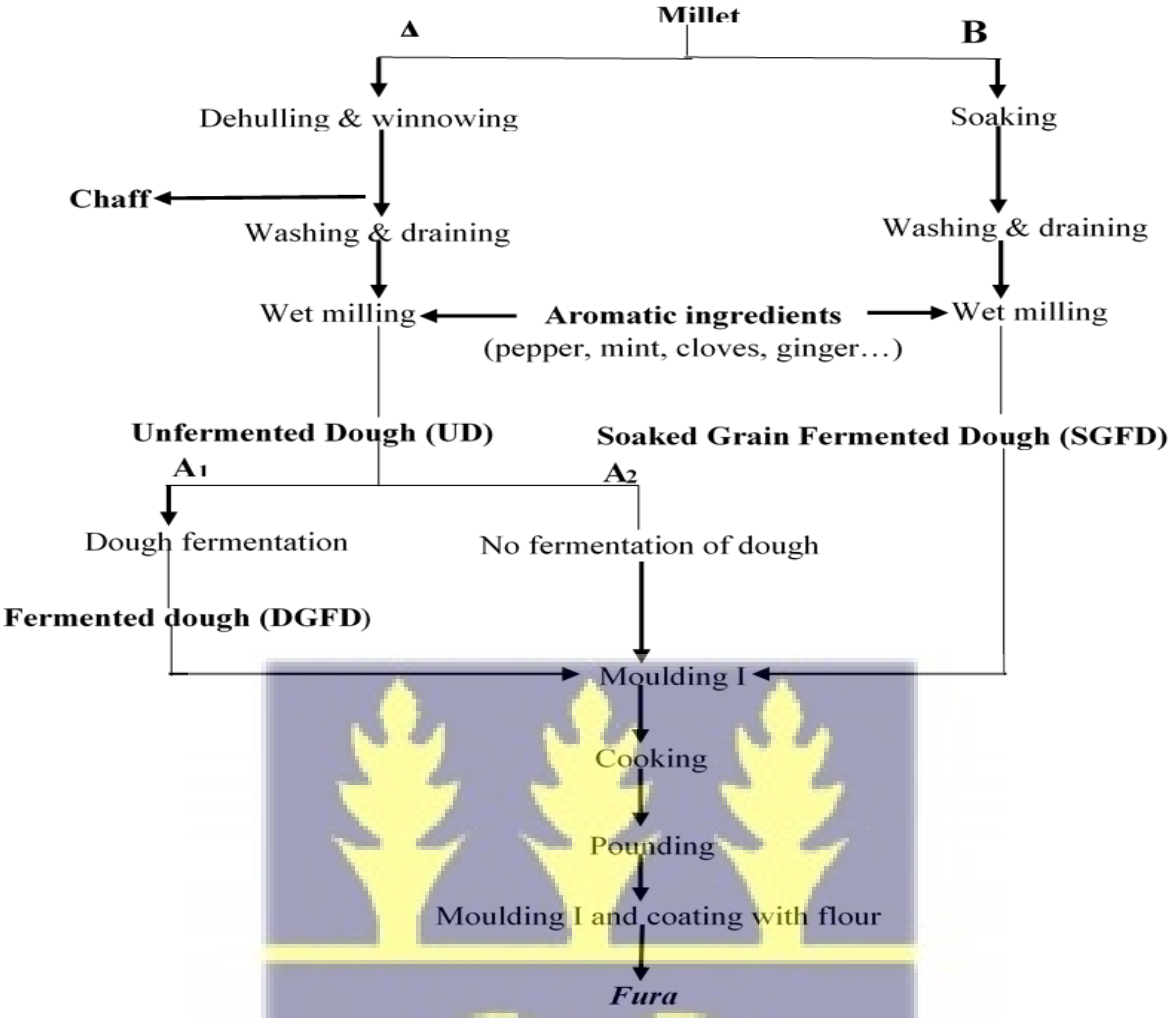


Figure 2: Flow chart for the traditional production of millet into *fura* (adopted from Owusu-Kwarteng et al., 2010)

2.3.1.2 *Maasa*

Maasa is a spontaneously fermented millet-based fried cake, maize or rice is sometimes used for its preparation. It is used mostly as an accompaniment for *koko*. According to Owusu-Kwarteng & Akabanda (2013), the traditional process of *maasa* preparation in northern Ghana consists of the following steps. The millet grains are steeped in water for 12 h, washed, drained and milled using an attrition mill. The dough is then divided; one-third of the dough is used to prepare a slurry, cooked into a pre-gelatinized meal and mixed with the remaining two-thirds to obtain a thick paste

which is fermented for 14 h. Servings of about 100 ml are fried in oil for about 5 min to obtain a millet-based cake known as *maasa* (Figure 3). Salt and pepper are sometimes added before frying (Dovlo, 1975). Ackaah-Gyasi (2010), however, reported that processors in Accra steep the millet grains in water for 8 - 24 h and ferment overnight (12-16 h). The division of the dough was not reported in this case. Water may be added to the paste after fermentation to soften it. Some processors also add sugar or mashed banana to sweeten before frying.

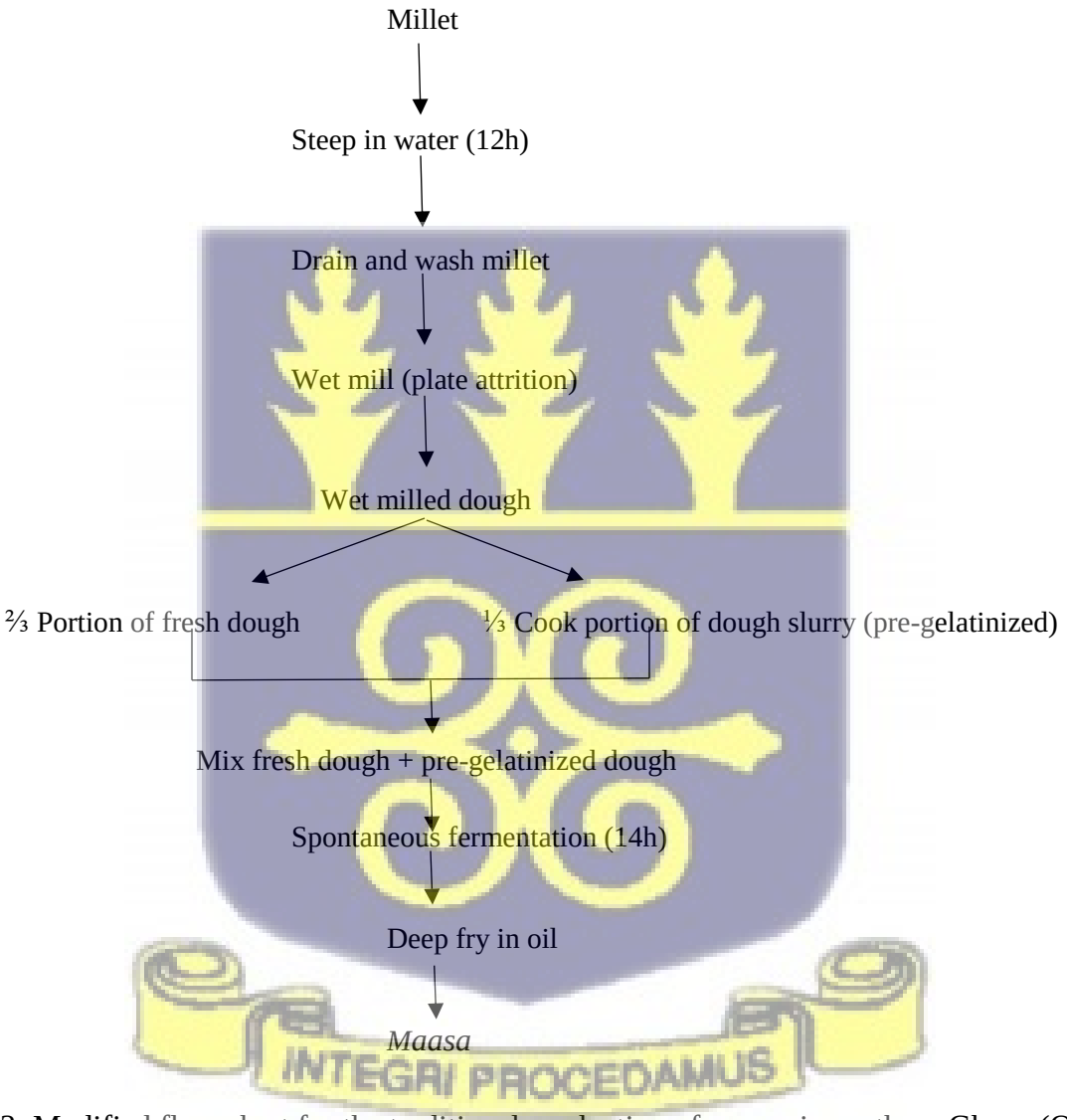


Figure 3: Modified flow chart for the traditional production of *maasa* in northern Ghana (Owusu-Kwarteng & Akabanda, 2013)

2.3.1.3 *Burkina*

Burkina, also called *brukina* is produced by a mixture of cow's milk and millet grits. In its production, the millet grains are steeped overnight and wetly milled. Water is sprinkled over the flour contained in a sieve, allowed to stand over boiling water for about 10 min and stirred to form grits. The grits are added to fermented cow milk and sugar may be added to taste. *Burkina* is consumed as a snack which is popularly sold in traffic intersections, markets, schools and other public places in Ghana. The production procedure is outlined in Figure 4 (Amoo-Gyasi, 2013).

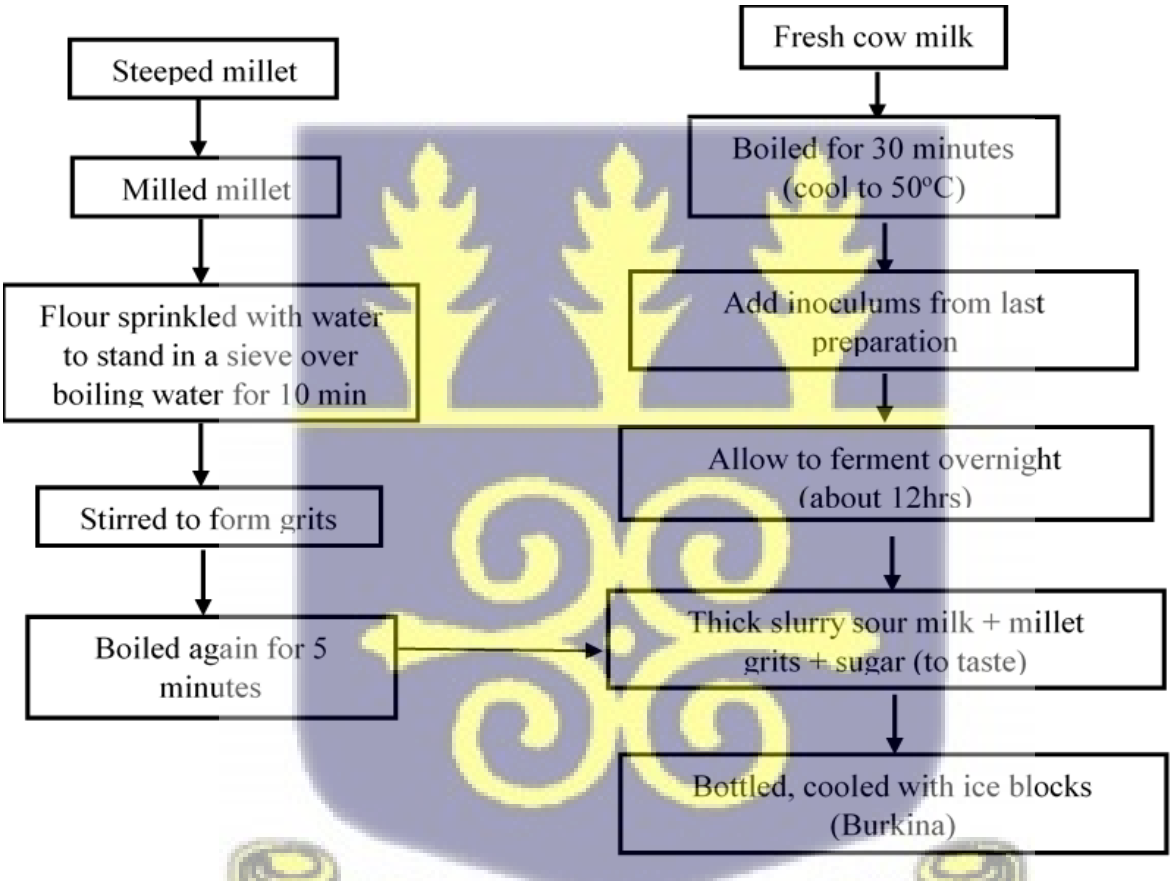


Figure 4: Flow chart for the traditional processing of *burkina* (Amoo-Gyasi, 2013).

2.4 Microbiota of fermented cereal foods

Bacteria and yeast species form the two main microorganisms associated with cereal fermentation (Corsetti & Settanni, 2007). The fermentation usually occurs in anaerobic conditions when there is an absence of oxidative phosphorylation to maintain Adenosine triphosphate (ATP) production by glycolysis. The two main organisms, bacteria and yeast involved in the fermentation undergoes lactic acid and alcoholic/ethanol fermentation. Some bacteria species may also be involved in acetic acid fermentation under aerobic conditions (Mani, 2018).

Lactic acid bacteria (LAB) are the main bacteria involved in cereal fermentation (Achi & Ukwuru, 2015). They produce lactic acid from the sugars present in the food naturally or the proliferation of LAB can be enhanced by the addition of lactic acid bacteria cultures (as starter cultures) to produce different fermented foods (Mani, 2018; Theron & Lues, 2010). They are a very important group of fermenting microorganisms that have GRAS (generally recognized as safe) status and thus are safe for consumption. They are widely used to preserve and or improve the nutritional qualities of the substrate as well as extend their shelf life in the form of starter cultures. As starter cultures, they have been accepted for the improvement of microbial safety by controlling the growth of pathogenic and spoilage organisms and improving the organoleptic properties through the production of metabolites, mainly organic acids, bacteriocins and many others (Schnürer & Magnusson, 2005; O'Sullivan et al., 2002; Messens & De Vuyst, 2002).

The principal LAB genera are *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Weissella*, *Oenococcus*, *Tetragenococcus* and *Vagococcus*. Although the genus Bifidobacterium is sometimes considered as part of the principal LAB genera because they share some typical features, they are phylogenetically unrelated and also have their distinctive model of sugar fermentation (Axelsson,

2004; Cousin, 1994). There are exceptions where for instance the bacteria of the genus *Gluconobacter* can produce acetic acid (vinegar) and bread yeast can produce carbon dioxide bubbles in the leavened dough (Scott & Sullivan, 2008).

LAB has a very complex nutrient requirement allowing them to ferment sugars using different pathways resulting in homo, hetero and mixed acid fermentation depending on the species involved (Hofvendahl & Hahn–Hägerdal, 2000; Cogan & Hill, 1993). They have two main hexose (glucose) fermentation pathways by which they metabolize:

i) Glycolysis is also known as Embden-Meyerhof-Parnas glycolysis pathway; produces only lactic acid as the end product of fermentation under standard conditions. This metabolism is known as homolactic fermentation which can be expressed as:

1 Glucose + $2NAD^+$ + 2ADA +2Pi → 2Pyruvate + 2NADH + $2H^+$ +$2ATP^+$

ii) 6-phosphogluconate also known as phosphoketolase pathway; produces lactic acid, ethanol, acetate and CO_2 as the end product of fermentation under standard conditions. This metabolism is known as heterolactic fermentation and can be expressed as:

Glucose +ADP +Pi → Lactic acid + Ethanol +CO2 + ATP

Lactobacillus (now *Limosilactobacillus)* is the largest genera included in LAB. It is a very heterogeneous group which is made up of obligate homofermenters (*Lactobacillus acidophilus, Lb. delbriickii, Lb. helveticus, Lb. salivarius*); facultative heterofermenters (*Lb. casei, Lb. curvatus, Lb plantarum, Lb. sakei*); and obligate heterofermenters (*Lb. brevis, Lb. buchneri, Lb. fermentum, Lb. reuteri*) (Kandler & Weiss 1986; Sharpe, 1981). *Lactobacillus* is the most acid-tolerant amongst the LAB group. Due to the health-promoting properties of the *Lactobacillus* species associated with the gut, they have enjoyed a lot of attention and are being promoted

globally as probiotics for the improvement of both human and animal health (Walter, 2008; Pfeiler & Klaenhammer, 2007; Schnürer & Magnusson, 2005). There has been a taxonomic rearrangement or reclassification of the genus *Lactobacillus* which before contained 261 diverse species as at March 2020. The reclassification has separated them into 25 genera. Some of which include *Liquorilactobacillus, Limosilactobacillus, Lactiplantibacillus, Latilactobacillus, Fructilactobacillus, Levilactobacillus, Schleiferilactobacillus, Amylolactobacillus, Holzapfelia* and *Acetilactobacillus* (Zheng et al., 2020).

Yeast is involved in ethanol or alcoholic fermentation. In the absence of oxygen, it converts one (1) molecule of glucose/fructose/sucrose into two (2) molecules of ethanol and two (2) molecules of carbon dioxide depicted in the equation (Mani, 2018):

$C_6H_{12}O_6 \rightarrow$ Pyruvate $\rightarrow 2C_2H_5OH + 2CO_2$

Yeast fermentation produces different compounds including alcohols, aldehydes, esters, lactones and terpenes (Stam et al., 1998; Janssens et al., 1992). Fermentation with fungi, in general enriches the food by the addition of fiber, proteins, vitamins, production of enzymes, and breakdown of anti-nutritive compounds (Bourdichon et al., 2012; Aidoo & Nout 2010). The main species of yeast associated with cereal fermentation is *Saccharomyces* but others like *Candida, Pichia, Debaryomyces, Kazachstania, Yarrowia, Hansenula* and *Trichosporon* have also been reported (Tamang et al., 2016). Mould species such as *Penicillum, Cladosporium*, *Fusarium* and *Aspergillus* have also been reported in cereal fermentation (Achi & Ukwuru, 2015).

Fermentation processes in Africa are mostly carried out spontaneously involving mixed cultures of a variety of microorganisms both desirable and undesirable. These may come from the raw materials, environment, and contact surfaces of utensils and so on. Conditions of incubation are then set to promote the proliferation of desirable types. However, because the natural microflora

in the raw material may differ from batch to batch, sometimes containing undesirable microorganisms, it may be difficult to produce fermented products that are safe and of consistent quality over a long period. Thus, there are high chances of product failure, contamination, and shorter shelf life as well (Bibek, 2004). Similarly, back-slopping involving the addition of a previously successful batch of fermented product to accelerate the fermentation of a new batch has also been reported as a potential source of contamination which can lead to foodborne diseases (Aka et al., 2014; Gadaga et al., 2004; Bibek, 2004; Antony & Chandra, 1999; Nout, 1992). The kind of bacterial population developed in fermented food depends on several factors. These include the fermenting matrix composition, temperature of incubation, pH, salt concentration and water activity (De Valdez et al., 2010).

The microbiota of fermented cereals in Ghana and other African countries has been extensively reported showing a mixture of homo and hetero-fermenting bacterial, yeast and in some cases, moulds occurring at different stages of the fermentation process. In *koko* and *koko* sour water from millet fermentation, Lei & Jakobsen (2004) reported the presence of *W. confusa*, *L. fermentum*, *L. salivarius* and *Pediococcus* spp. Other species observed in *koko* sour water were *L. salivarius*, *P. pentosaceus*, *P. acidilactici* and *L. paraplantarum*. API 50 CHL, Intergenic transcribed spacers (ITS)-PCR, restriction fragment length polymorphism (RFLP), restriction enzyme analysis with pulsed-field gel electrophoresis (REA-PFGE) and sequencing of the 16S rRNA gene were used in identifying these LAB. The dominant LAB involved in the processing of *fura* were identified using a combination of genotypic and phenotypic methods including (GTG)5-based PCR fingerprinting and 16S rRNA gene sequencing, multiplex PCR utilizing recA gene sequence comparison. The species includes *L. fermentum*, *L. reuteri*, *L. salivarius L. paraplantarum* and *Pediococcus* spp. Others including *Streptococcus* spp., *Leuconostoc* spp., *Enterococcus* spp. and *Issatchenkia*

orientalis were also identified. The yeast *S. cerevisiae, Pichia anomala, C. tropicalis, S. pastorianus, Yarrowia lipolytica*, and *Galactomyces geotricum* were also isolated (Owusu-Kwarteng et al., 2012; 2010). Using phenotypic and genotypic methods, Pedersen et al., (2012) reported the presence of *C. krusei, Kluyveromyces marxianus, C. rugose, C. fabianii, C. norvegensis* and *Trichosporan asahii* as the yeast population in *fura* and their potential probiotic properties. *W. confusa, L. brevis, P. acidilactici, Lc. lactis ssp lactis, Lc. rafinolactis C. krusei, C. albicans* and *C. membranifascians* were the additional microorganisms identified using phynotypic methods (Amankona, 2016). Using phenotypic methods, the identified LAB associated with *maasa* fermentation were *L. fermentum, L. acidophilus, Streptococcus* spp. *P. pentosaceus, P. damnosus, Lactococcus lactis* spp *lactis* and *Lc. lactis* spp *hordniae*. The population of yeast was dominated by *S. cerivisae* and *C. krusei*. The others were *C. norvengensis* and *Pichia farinose* (Ackaah-Gyasi, 2010). The population of LAB ranged from 10^7–10^9 CFU/ml whilst yeast count ranged from 10^3–10^5 CFU/ml. These were however not characterised and identified (Amoo-Gyasi, 2013).

Likewise in maize fermentation in Ghana, similar microflora was reported. The microbes involved in the fermentation of *nsiho* (white kenkey) were dominated by *L. fermentum, L. brevis, L. plantarum, Pediococcus pentosaceus, Pediococcus acidilactici, Debaryomyces* spp., and *Trichosporon* spp. *S. cerevisiae* and *C. krusei* were the dominant yeasts reported. The isolates were identified by determining their pattern of carbohydrate fermentation using the API 50 CHL kit and comparing them to the API database (Annan et al., 2015). Using phenotypic and genotypic methods, the dominant LAB microorganisms characterised during maize fermentation for Ga and Fanti *kenkey* production were *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus*

brevis and *Lactobacillus reuteri* while the dominant yeast species are *Saccharomyces cerevisiae* and *Candida krusei* (Obiri-Danso 1994, Jespersen et al., 1994; Halm et al., 1993;).

Similar microflora has been reported in fermented cereal foods from other African countries. In Burkina Faso, Abriouel et al., (2006) using culture-independent methods including sequencing of V3 region of the 16S rRNA gene reported the presence of *L. casei, L. brevis, L. fermentum, L. gasseri, Enterococcus sp.,* in dégué, a millet dough fermented food. Again in Burkina Faso, samples of fermented millet slurries for preparation of a guel known as Ben-saalga prepared at the laboratory scale or from small scale processing units were reported to contain various LAB (Humblot & Guyot, 2009). Isolates were cultured using MRS media, DNA and RNA extracted, the V3 region of the 16S rRNA amplified, pyrosequencing of the 16S rRNA gene amplicons performed using the 454 platforms. The sequence results were then compared with the Ribosomal Database Project. Some of the LAB genera reported included *Lactobacillus* (now *Limosilactobacillus), Pediococcus, Weissella, Streptococcus* and *Lactococcus.*

They also reported *L. plantarum/paraplantarum, Enterococcus sp., L. gasseri, L. acidophilus, Bacillus sp., L. reuteri,* and *L. casei* in poto poto, a traditional maize dough fermented food from the Republic of Congo. *L plantarum, L. helviticus, L. salivarus, L. casei, L casei, L. brevis, L. buchmeri, Leuc. mesenteroides, P. damnosus, S. cerevisiae* and *Schizosacchromyces pombe* were reported in Busa fermentation (Odunfa & Oyewole, 1998). Oguntoyinbo et al., (2011) used a combination of PCR-DGGE fingerprinting, sequencing of both V3 hypervariable regions and full length 16S rRNA genes to characterise *W. confusa, L. amylolyticus, L. delbrueckii* subsp. *bulgaricus, Lactococcus lactis* spp *lactis, L.fermentum, L. pantheris, Streptococcus lutetiensis, Strep. Gallolyticus* subsp *macedonicus, L. plantarum, L. vaccinostercus, Bacillus cereus* and *Clostridium perfringens* in fermented cereal ogi and kunu-zaki. In Benin, Vieira-Dalodé et al.,

(2007), used the internal transcribed spacer-PCR and 16S rRNA gene sequencing methods to characterise the dominant LAB in gowé, a fermented sorghum beverage. These they reported to include *L. fermentum, W. confusa, W. kimchii, L. mucosae, P. acidilactici* and *P. pentosaceus. Pichia anomala, Kluyveromyces marxianus, C. krusei* and *C. tropicalis* were the yeast reported after sequencing the D1/D2 domain of the 26S rRNA. Greppi et al., (2013) reported the yeast *S. cerevisiae, Clavispora lusitaniae, C. krusei, C. glabrata, Kluyveromyces marxianus, C. rugosa, Dekkera bruxellensis, Debaryomyces hansenii* in traditional cereal fermented foods again from Benin using both culture-dependent and independent methods.

2.5 Methods for identifying microorganisms

The total population of all the microorganisms in a food matrix usually referred to as microbiome plays an essential role in fermentation and other processes and that is the reason why an in-depth understanding of their taxonomy and communities is necessary for the improvement of these processes or mitigation of spoilage and contamination (Cao et al., 2017). Culture-dependent methods involving the phenotypic characteristic methodologies and culture-independent methods involving the genotypic characteristic methodologies are used.

2.5.1 Culture-dependent methods

Until recently, investigations of the microbiology of fermented foods, including porridges in Africa, have depended mostly on culture-dependent methods. Although this method according to Omar & Ampe (2000), is unable to detect microbial diversity, it has been employed in the identification of microorganisms associated with various fermented foods in Africa (Kigigha et al., 2016; Annan et al., 2015; Atter et al., 2014). These methods are selective for the enumeration of one type of organism at a time and based on the cultivation processes involving the use of

enrichment media or synthetic media that bears a resemblance to the natural state from which the organisms are isolated. This is followed by the isolation of the colonies on selective media after counting of colonies (Gugliandolo et al., 2011; Rantsiou & Cocolin, 2006). One of the major issues encountered at this stage is the selection of colonies for identification as closely related microbes often have identical colony morphologies, making it difficult to differentiate them. This process results in random isolation and missing out on other essential microbial constituents of the fermenting ecosystem (Rantsiou & Cocolin, 2006). Nonetheless, picking the colonies randomly helps to estimate the microbe's variety properly. Sometimes, some species may not grow at all in vitro (Head et al., 1998). This is followed by time-consuming biochemical characterisation or identification using indicators such as acid production capabilities, growth and survival at different temperatures of the organisms and many more. This is done using commercial kits such as Analytical Profile Index (API) or other biochemical tests and final confirmation tests (Gugliandolo et al., 2011; Rantsiou & Cocolin, 2006). The challenge however lies in the interpretation of the results as the positivity to a test, which is normally indicated by a change in the original colour of the medium, may in some cases, be subjective and inconclusive for the identification of an organism (Rantsiou & Cocolin, 2006). This method can only identify 0.1 % of a microbial community even though it is considered as the 'gold standard' (Cao et al., 2017). Although this method according to Omar & Ampe, (2000) is unable to detect microbial diversity in LAB, it has been extensively employed in the identification of microorganisms associated with various fermented foods mostly in Africa (Kigigha et al., 2016; Amankona, 2016; Annan et al., 2015; Atter et al., 2014). Currently, culture-dependent methods are complemented with molecular methods for the comprehensive characterisation of microbial isolates (Ercolini & Cocolin, 2014). One advantage with culture-dependent methods, is the isolation of viable microorganisms that can be

further characterised if they have good properties. With some culture-independent methods one may not have the isolates in hand.

2.5.2 Culture-independent methods

Molecular methods also known as culture–independent methods on the other hand circumvents most of the limitations and intrinsic biases encountered in conventional culture-dependent methods. It has demonstrated its effectiveness in providing a comprehensive overview of the total microbiota of fermented foods (Zhou et al., 2009). Different approaches are used to profile the microbiome. For total microbiota, one of such approaches is based on the direct extraction of DNA or RNA from the fermented sample. This is followed by library construction, conducting metagenomes sequencing and finally, gene analysis to determine the microbial diversity, ecology, phylogeny, activities etc. Another is based on the extraction of DNA or RNA from the fermented sample, the nucleic acids are then purified and subjected to amplification and profiling the population of microorganisms present in the food sample. Amplification may be by polymerase chain reaction (PCR) assay and real- time (RT)-PCR or other techniques (Cao et al., 2017; Gugliandolo et al., 2011; Ercolini & Cocolin, 2014; Rantsiou & Cocolin, 2006). DNA techniques for pattern analysis and typing include different fingerprinting options, Denaturing gradient gel electrophoresis (DGGE), Amplified fragment length polymorphism (AFLP), Ribotyping, Pulsed-field gel electrophoresis (PFGE), Temperature gradient gel electrophoresis (TGGE), Restriction fragment length polymorphism (RFLP), Repetitive element PCR (rep-PCR) and many others after which determination of the microbial diversity, ecology, phylogeny and activities can be investigated with the appropriate tools (Ercolini & Cocolin, 2014; Mohania et al., 2008). Another option after amplification is sequencing immediately without the need for pattern analysis or typing followed by determination of the microbial diversity with suitable tools.

2.5.3 The different sequence generation platforms

Sequencing of DNA to reveal the differences that exist in the sequence is the most appropriate method in distinguishing subtypes within an organism. It also allows for a faster means for organisms to be identified and cataloged (Abate et al., 2013). There are several of these sequencing methods which have evolved over the years from low throughput DNA fragment sequencing (first-generation sequencing) to high throughput next generation sequencing (NGS) otherwise referred to as the second generation and now third-generation sequencing (TGS) methods (Loman & Pallen, 2015; Hagemann, 2015). They differ in the DNA sequencing chemistries applied (Cao et al., 2017).

2.5.3.1 First generation sequencing

Sanger sequencing (Sanger et al., 1977) is a first- generation sequencing method also known as whole genome shotgun sequencing which is still extensively used and has undergone several transformations with key importance on producing sequencing reads at a faster and cheaper cost (Loman & Pallen, 2015; Metzker, 2005). It involves the use of DNA polymerase to synthesize several copies of the interested sequence in a single primer extension step whereby single-stranded DNA is used as a template with deoxynucleotide triphosphates (dNTPs) added which provides the nucleotide needed (A, arginine; C, cytosine; T, tyrosine; G, guanine) for extension (Hagemann, 2015; Gomes & Korf, 2018). A small amount of chain terminating 2´3´-dideoxynucleotide triphosphates (ddNTPs) for each of the nucleotides are also added to the reaction. At every nucleotide incorporation, there is the possibility of a ddNTPs to be added in place of a dNTPs as they both have an equal chance of attachment to the sequence. In the absence of 3´hydroxyl group, the extending DNA chain will be terminated in the long run by a ddNTPs yielding a DNA molecule of varying lengths (Hagemann, 2015; Gomes & Korf, 2018). The frequency of chain termination

in the sequencing reaction is determined by the dNTP/ddNTP ratio (Metzker, 2005). Over the years, there have been several variants of this sequencing method (Hagemann, 2015). One of current ones, is the automated Sanger sequencing where each of the ddNTP i.e ddATP, ddGTP, ddCTP and ddTTP is tagged with a specific fluorescent marker/dye which allows the base to fluoresce a particular colour based on the associated nucleotide of the ddNTP, when it attaches to the extending sequence. The colours of the nucleotide as indicated by the fluorescence produced are green for A, red for T, black for G and blue for C (Gomes & Korf, 2018; Metzker, 2005). As the marker/dye tagged fragments go through the region of detection, they get excited by the laser in the sequencer resulting in the production and emissions of the four different colours (Metzker, 2005). The fluorescent intensity produced is translated into peaks and detected by a laser within the automated machine used in reading the sequence (Gomes & Korf, 2018). The colour determination is the key method for allocating a base call whilst the order of the fluorescent fragments reveals the sequence of the DNA analysed (Metzker, 2005) which can be interpreted using base-calling softwares (Hagemann, 2015). It is also used to provide confirmation of variants that were unidentified by next-generation sequencing (NGS) methods and also to patch the coverage of regions poorly covered by NGS (Hagemann, 2015). About 800 to 1000 base pairs (bp) of read length are achieved with Sanger sequencing, with 99.99 % raw accuracy at a minimal cost (Zhou et al., 2010; Morozova & Marra, 2008). The in-vivo amplification of the DNA fragments to be sequenced which is achieved mostly by lengthy, labour intensive and host related-biases of cloning into a host seems to be the limitation of Sanger sequencing method (Hall, 2007).

2.5.3.2 Second generation sequencing

To address the challenges associated with the first-generation method, and improve on it, new sequencing methods started emerging commercially from 2005 (Barba et al., 2014; Guzvic, 2013;

França et al., 2002). These new methods known as second or next-generation sequencing methods (NGS) use amplified DNA as template (Munroe & Harris, 2010; Schadt et al., 2010). Originally, NGS was used for whole genome studies but now it's also used to study defined or selected regions of the genome (Koboldt et al., 2013). It determines DNA sequences using parallel sequencing of several small fragments of DNA simultaneously sometimes in multiple targeted genomic regions in the same run. These methods include DNBS (DNA nanoball sequencing); illumina (Solexa) Hiseq (large scale with higher throughput instrument) and Miseq (small scale with lower through-put) sequencing; Ion torrent and many others (Kulski, 2016; Serratì et al., 2016; Hagemann, 2015; Rizzo & Buck, 2012). They all differ from each other by way of differences that exist in the sequencing chemistry and methods used for signal detection (Serratì et al., 2016). The sequencing machines produce raw sequencing signals which are converted into short read data (base calling) or nucleotide bases using systems such as native raw data file formats, FASTQ format and others (Hagemann, 2015). Their challenges include the introduction of some errors during the DNA amplification process (Munroe & Harris, 2010; Schadt et al., 2010), the necessity to use bioinformatics tools requiring high-capacity storage, data analysis and data interpretation (Land et al., 2015; El-Metwally et al., 2013; Horner et al., 2010). Despite these challenges they are normally considered as 'high-throughput' methods due to the fast-sequencing speed, quantum of sequence data generated and the reduced costs (Voelkerding et al., 2009). In addition, it only requires a very small amount of DNA/RNA to run, and its sensitivity is far higher than Sanger sequencing (Serratì et al., 2016). Due to the high cost of NGS machines compounded with other need additions, several commercial sequencing service providers including Macrogene, Novogene, Illumina, Eurofins Genomics and others are available (Hagemann, 2015).

2.5.3.3 Third generation sequencing

Quest for further improvements in terms of cost reduction, simplification of the preparatory processes and many more on NGS methods culminated into the emergence of third generation sequencing technology which is very similar to NGS (Metzker, 2010; Schadt et al., 2010; Eid et al., 2009). It also uses parallel sequencing but of single DNA molecules as the template rather than the amplified DNA molecules as in the case of NGS (Munroe & Harris, 2010; Schadt et al., 2010). Some of the third generation sequencing methods include Single-molecule real-time (SMRT); Nanopore sequencing and Helicos sequencing. Some of them are designed as portable handheld devices that can be attached directly to a computer for DNA and RNA sequencing (Hagemann, 2015).

2.5.4 Techniques Applied in Microbiome Sequencing

2.5.4.1 16S ribosomal RNA /16S ribosomal DNA

The small subunit ribosomal RNA/DNA (16S rRNA/16S rDNA) macromolecules sequences of prokaryotes are used for microbial characterisation as both ribosomal RNA and DNA are present in all microbes (Wang & Qian, 2009; Lane et al., 1985). 16S rDNA is one of the most common culture independent techniques applied for microbiome analysis and one of the most common high throughput sequencing methods. It is based on the fact that prokaryotes in general and specifically bacteria have 16S rRNA genes which are sectioned into nine different highly conserved hypervariable regions (V1-V9). These hypervariable regions have conserved sequences which are used for species identification (Cao et al., 2017; Wang & Qian, 2009; Neefs et al., 1993; Lane et al., 1985). The challenge however is about the hypervariable regions to select as different reports favour the selection of different specific regions (Liu et al., 2008; Wang et al., 2007; Chakravorty et al., 2007). It was however reported (Claesson et al., 2010) from a study conducted on all the

hypervariable regions that in terms of efficiency, the V4/V5 was the best for the identification of food microbiome with reduced amplification bias as compared with V3/V4. Distinct PCR primers are therefore used for the amplification and sequencing of these hypervariable regions for the identification of bacterial taxonomy associated with a food matrix. For fungi on the other hand, mostly the 18S or 28S rDNA/rRNA gene sequences are used for their identification (Panzer et al., 2015; Feau et al., 2011). Based on the similarity of the nucleotide sequence result, the sequences are clustered into OTUs (Operational Taxonomic Units) and compared with those in databases for identification (Cao et al., 2017). It has the advantage of the availability of several bioinformatics tools such as QIIME (Quantitave Insights Into Microbial Ecology) for data analysis (Caporaso et al., 2010). Bacterial classifications using 16S rDNA sequencing mostly may not be identified beyond the genus level due to shorter reads obtained from NGS protocols most particularly from illumine platforms (Claesson et al., 2010).

Even though 16S rDNA has been determined for several bacteria species (Mechai et al., 2014), there are some reports that the sensitivity of using rRNA is higher. This is because rRNA content is more suitable for evaluating changes in metabolically active bacterial populations (Maukonen et al., 2003). Out of the different macromolecules including 5S rRNA that could be used for phylogenetic studies, 16S rRNA has been shown to be more precise in terms of their distribution, conservative nature and information density and so most recommended (Lane et al., 1985). Again, the use of rRNA was recommended for conducting taxonomic classification and phylogenetic studies (Wang & Qian, 2009).

Advancement in these techniques has seen the use of V3/V4 variable regions being used and, in some cases, the usage of only one hypervariable region for the identification of food microbiome.

For instance, DNA extracted from water samples were amplified using primers targeting the V3/V4 regions followed by illumina 16S rRNA sequence for microbial community determination (Nakatsu et al., 2019). Using the V3 region of the 16S rRNA gene only, Humblot & Guyot, (2009), identified the diversity of microbes in fermented pearl millet slurries. Diaz et al., (2019) also successfully used only the V4 variable region for microbiome identification in some African fermented foods. 16S rDNA sequence can also be aligned with 16S rRNA sequence from GenBank for bacterial identification using the sequence analysis tool known as Basic Local Alignment Search Tool (BLAST) reported in the bacterial diversity in fermented maize dough beverage, pozol from Mexico (Escalante et al., 2001).

2.5.4.2 Metagenomics

Metagenomics is a tool based on the isolation of nucleic acids directly from environmental samples and used to study microbial communities irrespective of their abilities to be cultured or not using isolation methods (Nazir, 2016). It is however unable to distinguish between viable microbial populations within a microbiome (Ercolini, 2013). It involves the direct isolation of DNA from a natural microbial habitat or environment such as marine water, soil, guts of vertebrates and invertebrates, fermented foods etc. The sequence-based analysis is on screening clones for conserved 16S genes mainly for identification and sequencing the complete clone to identify other available genes of interest or to look for phylogenetic anchors in the reconstructed genomes (Hoff et al., 2008; Riesenfeld et al., 2004). The functional-based analysis on the other hand is based on screening the DNA libraries for the identification of novel processes and proteins produced such as antibiotic production, enzyme activity, salt tolerance and others followed by the identification of the origin of the cloned DNA (Dinsdale et al., 2008; Sleator et al., 2008). This method unlike 16S rDNA-based approach is expensive but able to characterise bacterial to the species level and

provides in-depth information on the genes structure, evolutionary association and microbial community (Cao et al., 2017). This technique provides a means for the advancement of novel genes, natural products, enzymes, antibiotics, bio-surfactants, bioactive compounds as well as processes that could impact industrial and biotechnological applications (Nazir, 2016; Warnecke & Hugenholtz, 2007).

2.5.4.3 Whole Genome Sequencing

Whole genome sequencing (WGS) involves the complete sequencing of an organism's whole genome thereby providing a detailed collection of the organism's genetic variations (Ng & Kirkness, 2010). It is also known as complete genome sequencing, full genome sequencing, or entire genome sequencing. This method is the most advanced currently and has also undergone a lot of improvement. It provides information on the organism's complete DNA sequence. It enables rapid characterisation and accurate identification of microbial strains, provide in-depth information on the microorganisms, origin, subtyping, a better understanding of their diversity, capabilities, roles, phylogenetic relationships, predict antimicrobial resistance (AMR) genotypes, metabolic potential, susceptibility to diseases, and clues on other relevant novel functions. This information are provided without the need to conduct any further analyses in the laboratory. (FAO, 2016; Douillard & De Vos, 2014; Siezen et al., 2004). Knowledge of WGS is influential in genome mining and prompt selection of precise features (Douillard & De Vos, 2014). It provides the characterisation of microorganisms with a high degree of precision within few days and the data can be easily stored in repositories, shared, analyzed and reanalyzed or mined at any time (FAO, 2016). Its application in characterizing microbes in food fermentation is limited. It has however been extensively used in foodborne pathogen typing in several outbreak investigations in several countries and for routine surveillance (Smith et al., 2020; Nouws et al., 2020; FAO, 2016).

Challenges such as standardization and harmonization of workflow including the type of kits used for DNA extraction and bioinformatics workflow must be considered to exploit its full potential (Bogaerts et al., 2021; Nouws et al., 2020). Sanger sequencing can be used for whole genomes. The difference here is that Next Generation Sequencing for Whole genomes have high throughput which also makes it time efficient and cost effective for whole genomes compared to Sanger sequencing.

2.6 Nuclear magnetic resonance spectroscopy for metabolites detection

The transformation process that occurs during food fermentation results in structural changes, formation, modification and degradation of the compounds involved, thereby resulting in either an upsurge or reduction in the compounds involved. Comprehensive molecular profiles of several of the biochemical, physicochemical and structural metabolites produced in such fermented foods can be identified through food metabolomics, also known as foodomics, in a single run (Adebo et al., 2017; Hu & Xu, 2013; Cifuentes, 2009). Food metabolomics or foodomics which is thus the study of several metabolites in food under specific conditions and time through the application of omics technology includes sample preparation, extraction, data acquisition and its analysis (Adebo et al., 2017). Gas chromatography-mass spectrometry (GC-MS) is one of the common analytical platforms used for studying metabolites from fermented cereals (Adebo et al., 2021). The use of high-performance liquid chromatography (HPLC) has also been reported in some studies for organic acids and volatile compounds analyses (Mugula et al., 2003a).

Metabolomic variations can also be studied through analytical tools such as the Nuclear Magnetic Resonance (NMR) spectroscopy together with multivariate data analysis such as principal component analysis. The NMR spectroscopy provides a simple structural analysis attained from

the metabolites signals and their intensity relative to the molar concentration can give information on the quality and quantity of the identified metabolite (Kim et al., 2010). Separation and chemical modification of samples is not needed in NMR measurements and for that matter, the method provides information on chemical components in complex mixtures quickly and directly (Lu et al., 2016). ^{1}H NMR is considered as the most useful amongst NMR-based comprehensive analyses such as ^{13}C NMR, ^{31}P NMR spectra due to its informative spectral patterns and high-throughput acquisitions (Wei et al., 2010). It has been used to identify many chemical compounds in fermented foods such as soy sauce, yoghurt and wine in developed countries (Lu et al., 2016; Vázquez-Fresno et al., 2015; Li et al., 2014).

2.6.1 Metabolites of fermented foods

Metabolites produced by fermenting microorganisms, mainly bacteria and yeast during cereal fermentation are beneficial in several ways. Production of some of these metabolites are used in accessing the technological and probiotic potential of fermenting microorganisms (Adesulu-Dahunsi et al., 2018; Owusu-Kwarteng et al., 2015; Mechai et al., 2014). These metabolites impart positively to help improve the safety of fermented food and they include: organic acids, bacteriocins, exopolysacharides, volatile compounds, hydrogen peroxide, enzymes, carbon dioxides and diacetyl

2.6.1.1 Organic acids

During fermentation of in cereals by lactic acid bacteria, lactic and acetic acids are the key organic acids produced. They can also produce other organic acids such as propionic, succinic, formic, citric, caproic, butyric and valeric acids. These organic acids have GRAS status and can also be commercially produced by chemical synthesis (Madigan et al., 2012; Theron & Lues, 2010; Nes

& Johnsborg, 2004; Corsetti et al., 1998). Lactic acid is partially lipid soluble which allows it to diffuse slowly through the cell membranes (Gravesen et al., 2004). Its inhibitory potentials against several microorganisms are through the ability of the LAB to synthesize and excrete enough quantities of lactic acid which result in the reduction in pH of the fermenting matrix (Theron & Lues, 2010; Herreros et al., 2005; Davidson et al., 1995). The species of LAB available in the fermenting matrix, the growth conditions and the food composition determine the type and quantities of organic acids produced during any fermentation process (Ammor et al., 2006; Lindgren & Dobrogosz, 1990). The organic acids permeate through the membrane of the target organism and reduce the pH of its cytoplasm and consequently halts the metabolic activities of the target organism (Piard & Desmazeaud, 1991). The various organic acids produced during the process impact stabilizing and preservation properties on the food in addition to flavour enhancement. These in addition to other qualities ultimately improves the overall sensory attributes of the food, reduction in preparation time and fuel requirement cannot be overemphasized (Arena et al., 2016; Min et al., 2007; Gomis 1992).

2.6.1.2 Bacteriocins

Bacteriocin is another essential antimicrobial substance produced by some bacteria in general but more specifically by LAB. They have been described as peptides that are extracellularly released and capable of inhibiting the growth of other closely related bacteria with activity similar to antibiotics (Hernández-González et al., 2021; De Vuyst & Leroy, 2007). Archaec, Gram- positive and Gram-negative bacteria produce bacteriocins (Savadogo et al., 2006) and are often used in combination with other antimicrobial substances like organic acids (Theron & Lues, 2010). *Lactobacilli*, *Lactococci*, *Leuconostocs*, *Pediococci*, and *Streptococci* are some of the bacteriocin-producing genera from Gram positive bacteria (Nilsen et al., 2003). Bacteriocins have been

classified using different schemes. Some of the schemes grouped them into 2 categories that is, lantibiotics (class I) and non-lanthionine containing bacteriocins (class II) (Cotter et al., 2005). Others grouped them into four categories i.e. Class I to IV (Rattanachaikunsopon & Phumkhachorn, 2010; Jeevaratnam et al., 2005). The groupings are based on their molecular mass, sensitivity to enzymes activity, chemical structure, modified amino acids content and activity mechanism (Bodaszewska-Lubas et al., 2012). Those from Gram positive bacteria including LAB largely belong to Classes I and II (Jeevaratnam et al., 2005). Class I bacteriocins are small peptides that are less than 5 kDA containing heat stable amino acids ranging between 19 to 50. They undergo post translational modifications leading to the formation of the unusually modified amino acid known as lanthionine and methyllanthionine. Class II bacteriocins are less than 10 kDA and do not contain the modified amino acid lanthionine. They are also pH and heat resistant (Hernández-González et al., 2021; Nilsen et al., 2003). Bacteriocins like Nisin from *L. lactis* spp, Enterolysin from *Enterococcus faecium*, Plantaricin from *L. plantarum* spp, helveticin from *L. helviticus* are some of the well characterised bacteriocins in class III. They have larger molecular weight containing more than 30 kDA and are heat labile (Zacharof & Lovitt, 2012). Class IV bacteriocins are very complex containing carbohydrate moieties as well as lipids and usually not preferred because of possible inclusion of unpurified bacteriocins (Saeed et al., 2014). Bacteriocin-producing LAB is gaining a lot of attention globally especially in Europe, America and Asia due to their GRAS status, prospective use as natural food additives for preservation and solution to the incidence of food spoilage and foodborne infection. In addition, they are deemed to have therapeutic antibiotics properties for usage as natural agents to treat systemic diseases and can replace antibiotics as active agents against multiple drug-resistant pathogens (López-Cuellar et al., 2016; Perez et al., 2014; Cotter et al., 2013; van Heel et al., 2011; Diop et al., 2007; Cleveland et al., 2001). Some well-characterised bacteriocins from LAB include Nisin, Pediocin A and AcH,

Helveticin J and Leucocin (Soomro et al., 2002). They are often used in combination with other antimicrobial substances like organic acids (Theron & Lues, 2010). They are active against a wide range of spoilage and pathogenic microbes (Rattanachaikunsopon & Phumkhachorn, 2010).

2.6.1.3 Exopolysaccharides (EPS)

Extracellular polymeric compounds composed mainly of polysaccharides, known as Exopolysaccharides (EPS), are also secreted into the fermenting matrix (Mollakhalili Meybodi & Mohammadifar, 2015). They are mostly classified into two groups in terms of their composition as homo-polysaccharides which are made up of only one type of monosaccharide (glucose or fructose for example), produced mainly by the *Weissella* genera. The second group is the hetero-saccharides of repeating units of different types of monosaccharides produced by mesophilic and thermophilic LAB such as *L. sakei* (Oleksy & Klewicka, 2018; Sanalibaba & Çakmak, 2016). Other monosaccharides may include amino sugars, galactose, pentoses, rhamnose and hexoses (Kumar and Mody, 2009). Conditions such as temperature, pH, duration of incubation during fermentation affect the yield as well as the composition of EPS produced by LAB (Caggianiello et al., 2016). They are made up of long chain polymers with varying molecular weights and structures. Some microbial EPS include dextran originating from LAB, gellan from *Pseudomonas elodea*, xylinan from *Acetobacter xylinum* and xanthan from *Xanthomonas campestris*. EPS from LAB affect the rheological qualities of the fermenting matrix resulting in a ropy and viscous end product. The synthesis of EPS by LAB strains are mostly applied at low concentration and imparts health benefits to the consumer (Caggianiello et al., 2016; Mollakhalili Meybodi & Mohammadifar, 2015). They contribute to the improvement of the nutritional properties, sensory characteristics, thickening agents, viscosity, texture and stability of fermented foods (Oleksy & Klewicka, 2018; Mollakhalili Meybodi & Mohammadifar, 2015; Górska et al., 2007; Ruas-

Madiedo et al., 2002; De Vuyst et al., 2001; Cerning, 1994). EPS producing LAB are mostly probiotic. Their probiotic properties and health benefits including antitumoral and blood cholesterol lowering effect have also been reported (Caggianiello et al., 2016).

2.6.1.4 Diacetyl

According to the National Institute of Health (2015), diacetyl (2, 3-butanedione) is a very volatile aromatic compound having a vapor pressure of 56.8 mm Hg at 25 ºC. Humans have been exposed to diacetyl from the beginning of civilization because it is present naturally in several foods, giving them an appealing butter-like aroma and formed naturally through the action of microorganisms during fermentation. It generally improves the aroma of foods and is an essential flavour compound in margarine, yoghurt, butter, and cheese (Clark & Winter, 2015; Owens et al., 1997). It is one of the volatile compounds with a GRAS status formed through carbohydrate catabolism during LAB fermentation. Its produced by some species of LAB including *Lactobacillus* (now *Limosilactobacillus*), *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Oenococcus* and sometimes used as a food additive (Ammor et al., 2006; Bartowsky & Henschke, 2004; Toldr´a 2004; Ugliano et al., 2003; Birkenhauer & Oliver, 2003). During fermentation, the diacetyl content in a product may increase due to its formation by LAB as the fermentation progresses and decease due to its conversion into other fermentation end products (Attaie, 2009). It is also used as a food additive due to its GRAS status (Clark & Winter, 2015; Birkenhauer & Oliver, 2003; Owens et al., 1997). Both Gram positive and negative bacteria including *E. coli*, *Salmonella*, *Listeria* and *Yersinia* are inhibited by diacetyl (Ammor et al., 2006).

2.6.1.5 Carbon dioxide

The production of carbon dioxide (CO_2), one of the antimicrobial products of heterolactic fermentation plays a very important role in the entire fermentation process creating an anaerobic environment that is toxic mostly for aerobic microorganisms (Lindgren & Dodrogosz, 1990). It is generally also very effective for prolonging the shelf life of foods that are perishable by impeding bacterial growth (Daniels et al., 1985). The quantity produced in a fermentation matrix depends on the types of species and the acidifiers such as *L. lactis* that are present (Monnet et al., 2002). It causes a reduction in the pH inside and outside the cell of the microorganism. It therefore reduces their survival and subsequent inhibition or elimination (Achi & Ukwuru, 2015; Lindgren & Dodrogosz, 1990). The general effect of CO_2 is to increase the lag phase as well as the generation time of the spoilage organism. One of the general mechanisms proposed for its bacteriostatic effect is that its presence in the cells poses a negative effect on different enzymatic and biochemical pathways which imposes stress on the system and slows down their growth rate (Daniels et al., 1985). Produced CO_2 in a fermenting substrate can enhance its safety, as reported with kimchi, a fermented Chinese mixture of vegetables, grains, spices and salted anchovies (Park, 2018). Carbon dioxide gas is also used to protect foods from oxidation, sometimes in combination with nitrogen in modified atmosphere packaging (Lee, 2016). Its effectiveness usually increases with its concentration but the challenge with this is the likelihood of creating favourable conditions for pathogenic organisms such as *Clostridium botulinum* to thrive (Daniels et al., 1985).

2.6.1.6 Hydrogen peroxide

LAB produces hydrogen peroxide (H_2O_2) as one of the inhibitory substances against spoilage and foodborne pathogens during fermentation (Ito et al., 2003) through oxidation of sugars or other similar compounds (Kot et al., 1996). It can be produced mainly on different carbon and nitrogen

sources over a wide range of temperatures with an optimum at 37 ºC and pH of 5.5 (Enitan et al., 2011). LAB produces hydrogen peroxide due to the action of nicotinamide adenine dinucleotide (NADH) peroxidase and or flavoprotein oxidases (Collins et al., 1980). Thus, its accumulation mainly occurs in strains that lack the core hydrogen peroxide scavenging enzymes, that is, catalase and NADH peroxidase (Hertzberger et al., 2014). It is a precursor for the production of free radicals including superoxide (O2-) and hydroxyl (OH-) which damage DNA and are bactericidal (Ammor et al., 2006). The hydrogen peroxide produced can accrue to a level that then becomes inhibitory to the proliferation of other organisms in culture and destroys sensitive organisms by oxidation of the cellular materials as well as the basic molecular structure of the cell protein (Zalán et al., 2005). It has inhibitory effect against *Listeria monocytogenes, Clostridium botulinum* type E, *Brochthrix, Lactobabillus* and *Pseudomonas* (Ito et al., 2003). *L. johnsonnii* NCC 533 is a typical H_2O_2 producing LAB which has been proposed to also have probiotic properties (Hertzberger et al., 2014; Pridmore et al., 2008). Adesokan et al., (2010) reported the production of H_2O_2 at different quantities ranging from 0.024 g/L - 0.016 g/L under different cultural conditions by isolates of *L. brevis, L. fermentum, L. plantarum, L. delbrueckii* and *Leuconostoc Mesenteroides* isolated from some traditional Nigerian fermented foods.

2.6.1.7 Enzymes

Enzymes such as proteinase, peptidase, amylase, lipase, mannase, cellulase and catalase are involved in fermentation, with the responsibility of degrading complex macronutrients in raw materials into simpler forms (Tamang et al., 2016; Marsh et al., 2014; Nout & Rombouts, 2001). Starch, the main macronutrient in cereals is mostly degraded by the enzyme amylase which are found in microbes associated with plants and animals (Nazir, 2016). Different amylases including iso-amylase, alpha-amylase, beta-amylase and amyloglucosidase are all responsible for starch

degradation simultaneously during cereal fermentation (Nout & Rombouts, 2001). This results in the production of reducing sugars such as glucose and maltose which are utilised by the fermenting microorganisms for growth and nutrients for the consumer (Iyer & Ananthanarayan, 2008). Aside from fermentation, amylases have potential in the pharmaceutical industries for starch hydrolysis (Nazir, 2016). Enzymatic activities also cause cell wall degradation leading to softening of cereals which also improves the sensory attributes of fermented cereals (Poutanen, 2020).

2.7 Other benefits of fermented cereals

Cereals on their own are regarded as functional foods due to the nutrients they provide and the beneficial health effect on consumers (Achi & Ukwuru, 2015). When they are fermented, the functional microorganism involved enhances the functionality of the fermented cereal food. These functional microorganisms transform the chemical components of the substrate during the process to degrade toxic and antinutrient compounds, enhance nutrients bioavailability, produce antimicrobial compounds to impart bio-preservative properties, improve the safety of the food, improve the sensory attributes and impart health benefits to the consumer (Tamang et al., 2016).

2.7.1 Antinutrients reduction

Cereals contain micro and macro-nutrients, phytochemicals as well as antinutrients like phytate, tannin and polyphenol, which bind together forming insoluble complexes in the food and reduces the release or bioavailability of the nutrients to the consumer. These can cause micronutrient malnutrition and mineral deficiencies (Samtiya et al., 2020; Nkhata et al., 2018). The fermentation process generally disrupts these complexes and reduces the antinutrients levels (Nkhata et al., 2018; Sindhu & Khetarpaul, 2001). Fermentation provides the optimum pH conditions enabling endogenous enzymes such as amylase, phytase, pullulanase and other glucosidases to degrade

these antinutrients. For instance, phytate is usually stored as phosphate and inositol in plant seeds such as cereals which forms complexes with zinc, calcium, iron and affect lipid and protein utilisation. Phytase breaks down phytate in the form of complexes with polyvalent cations including magnesium, calcium, iron and zinc. The bioavailability of these soluble minerals then increases with the reduction of phytate (Samtiya et al., 2020; Nkhata et al., 2018; Kumar et al., 2010; Blandino et al., 2003; Nout & Ngoddy, 1997). Similarly, the levels of tannins and polyphenols are reduced as a result of the activities of tannase and polyphenol oxidase which are present during the fermentation (Sindhu & Khetarpaul, 2001; Nout & Ngoddy, 1997). Thus, phytates, tannins, polyphenols and other undesirable compounds can be detoxified through fermentation which provides an effective means to enhance the nutritional level and reduce mineral deficiency among consumers especially children in developing countries (Samtiya et al., 2020; Sharma & Kapoor, 1996). Reduction of tannins, phytic acids, phenolic compounds and mineral binders were reported in traditional fermented Zimbabwean finger millet porridge (Gabaza et al., 2019).

2.7.2 Probiotics and prebiotics

Probiotics have been defined as "live microorganisms which when administered in adequate amounts confers health benefits to the host" (FAO/WHO, 2002). The genera *Lactobacillus* and *Bifidobacterium*, yeast, *Bacillus* and *Propionibacterium* are used as probiotics (Nagpal et al., 2007; Felis & Dellaglio, 2007; Jan et al., 2001). The different inhibitory substances including lactic acid, acetic acids, citric acids, hydrogen peroxide, bacteriocin, diacetyl and many more produced by LAB during fermentation creates antagonistic environment for foodborne pathogens and spoilage organisms accounting for their extensive usage as probiotics (Nagpal et al., 2012).

A potential probiotic specie must not be pathogenic, carcinogenic or allergenic. It should be tolerated by the immune system and must be capable of colonization and proliferation in the intestinal tract (Ohashi & Ushida, 2009; Toma & Pokrotnieks, 2006). Acidification, amylolytic potential, tolerance to low pH, bile salt hydrolysis, tolerance to gastric juice during transit, cell surface hydrophobicity and many more key attributes are tested in potential probiotic isolates (Adesulu-Dahunsi et al., 2018). Some of the well-studied commercial species available and used by various industries include *L. acidophilus* R0011, *L. casei* Shirota, *L. fermentum* RC-14, *L. johnsonii* La1, *L. reuteri* SD2112, *L. plantarum* 299V, *S. cerevisiae (boulardii),* and *L. paracasei* F19 (Nagpal et al., 2012; Aureli et al., 2011). They inhibit the colonization of pathogens through their attachment to epithelial cells as well as their physical blocking of the pathogen's capability to adhere (Gueimonde et al., 2006). The intestinal tract contains food at various stages of digestion, digestive ferments as well as solid and liquid waste. There are also wide ranges of both beneficial and harmful microorganisms whereby the beneficial ones contribute to the production of essential vitamins, sugars, dietary fiber, fatty acids, amino acids, breakdown and destruction of toxic compounds that may have been consumed and most importantly breakdown of food. Irrespective of the health status of an individual, the different microbes compete to establish their dominance in the warm and moist ecosystem for their existence and proliferation. A healthy balance is however achieved if a ratio of 85 % of beneficial bacteria to 15 % of harmful bacterial is maintained in the intestinal tract (Savadogo et al., 2006).

Intake of probiotics from whatever source is therefore necessary and endorsed (Draper et al., 2017). Supplementation of foods with probiotic microorganisms is considered safe and approved by regulatory bodies (Ricci et al., 2018). They are supplemented into various food products such as breakfast cereals, formulations for infants, nutrition bars, ice cream, fermented milk, fermented

yoghurts, fruit juices, fermented dairy desserts, capsules, tablets and cosmetic products (Nagpal et al., 2012; Nagpal & Kaur, 2011; Nagpal et al., 2007; Shah, 2000). These foods must contain an adequate number of probiotic microorganisms to effect the required health benefits to the consumer or user which has been proposed to be roughly 10^{11} Colony Forming Unit (CFU) or at least 10^{7} CFU/100 g or ml (Aureli et al., 2011; Nagpal et al., 2012; Ross et al., 2002).

Exposure to probiotics has been through fermented foods which are very synonymous with most African fermented foods (Nagpal et al., 2007). Even though a lot of research has been carried out on the identification of the fermenting organisms and their diverse probiotic potentials (Halm et al., 1993), only a few of these have dealt with ascertaining their fitness to be classified as probiotics conclusively and the required dosage to confer health benefits (Anukam & Reid, 2009; Ghrairi et al., 2004). That notwithstanding, there are reports of its usage in the prevention and treatment of several diseases associated with the gastrointestinal tract (GIT) including diarrhoea (Lei et al., 2006). However, in advanced countries where extensive research has been conducted on them, their benefit is enormously outlining some with strain -specific benefits that may not apply to all strains. They can improve the immune system, digestive system, stool frequency and consistency, prevent and reduce respiratory infections, lessen discomfort in the GIT, reduce infection and allergies in children, improve food safety, prevent and treat digestive inflammation and other diseases (Allen et al., 2014; Aureli et al., 2011; Zanello et al., 2011; McFarland, 2007).

One of the means to increase the population of probiotics in the intestine or colon is by supplying them with selective natural occurring carbon and energy sources which provides the added advantage over other bacteria in the ecosystem. These selective carbon and energy sources which are non-digestible food ingredients in the form of fibre are referred to as prebiotics (Pandey et al.,

2015). Non-digestible oligosaccharides (inulin, oligo-fructose, galacto-oligosaccharides), non-digestible carbohydrates, unrefined cereals are some of the compounds of prebiotics (Pandey et al., 2015). Currently, any raw material that is utilized by microorganisms in a host and imparts some health benefits to the host is also regarded as prebiotics (Gibson et al., 2017). These include whole grains, fruits and vegetables. Depending on the extent of fermentation, fermented cereal products may also be considered as prebiotics as they contain soluble fibers, peptides, resistance starch and phenolic compounds (Tsafrakidou et al., 2020).

These prebiotics also possess many health benefits including relief from bowel disorders such as decreasing the occurrence and extent of diarrhea (Peña, 2007). Orally administered prebiotics also promote metabolic health in dealing with obesity and type 2 diabetes, skin health, constipation, and general gut health (Gibson et al., 2017). When suitable prebiotics are used together with probiotics, they can improve the viability and growth of the probiotics, such an association having a synergistic effect is termed symbiotic (Pandey et al., 2015; Nagpal et al., 2012). It also promotes the attachment and growth of new additions of probiotic strains (Nagpal et al., 2012).

2.7.3 Health benefits

The functional value of cereals is improved after fermentation resulting in several nutritional and health benefits. Antimicrobial compounds, aromatic compounds, minerals, essential amino acids, vitamins and many others are produced during fermentation as well as the synthesis of biologically active compounds which are well known for their nutritional benefits, health benefits and disease prevention (Balli et al., 2019; Ray et al., 2016; Tamang et al., 2016; Marsh et al., 2014). The health benefits include the prevention of hypertension, intestinal infections, diabetes, cancer, gastrointestinal conditions, reduction in cholesterol levels, and cardiovascular diseases (Tamang

et al., 2016). Reduction in toxicity and digestibility of gluten is improved as a result of fermentation (Rahaman et al., 2016; Gänzle et al., 2008; De Angelis et al., 2006). Other health benefits ensue from bacteriocins produced during fermentation having an antimicrobial effect; bioactive peptides, which exhibit antioxidant, antimicrobial, antiallergenic, and blood pressure lowering effects (Zannini et al., 2012; Vasiljevic & Shah, 2008). Traditionally in Ghana, shingles are locally treated by smearing fermented maize dough on the body (Mensah, 1997). Reduction of bacterial contamination can also be achieved through fermentation which could also help to decrease the prevalence of diarrheal illnesses (Mensah et al., 1990). Cooking of these cereals as is the practice in Ghana and most African countries, is believed to affect this property by reducing the antimicrobial effect on pathogens. Nonetheless, significant inhibition of the pathogens still existed as reported during the cooking of fermented maize dough into porridge (Mensah, 1997; Mensah et al., 1991).

2.8 Starter cultures

Spontaneous fermentation of cereals is mostly associated with mix population of microorganisms originating from the substrate, environment, utensils and other sources which could include potential pathogens. Conditions are then set for the proliferation of the microorganisms. Although a succession of microbes occur, the best-adopted strain to the environment in the fermenting matrix with the maximum level of growth takes over the fermentation process. This results in inconsistencies in product quality, safety, potential product failure and food safety concerns (Fessard & Remize, 2017; Ogunremi et al., 2017; Bibek, 2004; Holzapfel, 2002). Inoculation of a previously successful fermentation in the form of dough or beverage to a new batch of substrates for fermentation known as backslopping is also practiced in most traditional processes in Africa. Similar to spontaneous fermentation, it also has the same challenges (Brandt, 2014; Bibek, 2004).

The surest means to control and optimize such process to achieve reproducible, predictable, safer, quality products, as well as in the quantities that will satisfy consumer demands, is by using well characterised starter cultures for fermentation (Kimaryo et al., 2000). Starter culture involves inoculating the substrate with well characterised single or multiple strains of cultures to regulate and hasten the fermentation process (Fessard & Remize, 2017). Starter cultures are presumed safe by the Food and Drugs Administration (FDA) as well as the European Food Safety Authority (EFSA) (Bourdichon et al., 2012). Again, they have GRAS status and are safe for consumption as functional foods and use in the implementation of quality assurance measures (Mokoena et al., 2016; Welman & Maddox, 2003; Jespersen 2003). A prospective strain must excell in four main criteria for a starter which are pinned on the nutritional, safety, technological and sensory properties (Fessard & Remize, 2017). They must be capable of tolerating stressful conditions and secrete key metabolites during fermentation to ensure process control and predictability (Alfonzo et al., 2013). Quick proliferation and acidification rate, development of flavour compounds, anti-fungal compounds, improved nutritional content, bacteriocin production, hydrogen peroxide, diacetyl and many others are of essence (Fessard & Remize, 2017; Varsha & Nampoothiri, 2016; Rattanachaikunsopon & Phumkhachorn, 2010). The screening for selection of desirable strains must involve the use of high through-put methods presenting functional characteristics and must be compatible with the matrix. This will yield products with assured quality, safety, wider acceptance, enhanced shelf life, easy-to-cook form and improve the safety and quality of the final product (Ogunremi et al., 2017). The selection process involves isolation and *in-vitro* selection, laboratory scale validation of the isolate and finally, factory scale validation of the isolate (Fessard & Remize, 2017; Bevilacqua et al., 2012).

In cereal fermentation, lactic acid bacteria (LAB) and fungi mainly yeast, are mostly used as starter cultures (Brandt, 2014). In Africa, they form the main fermenting microorganisms in most indigenous cereal fermented foods (Humblot & Guyot, 2009; Vieira-Dalodé et al., 2007, Halm et al., 1993). The LAB confer rapid acidification properties and yeast confers a high alcoholic fermentation rate (Singh et al., 2015; Galati et al., 2014). Starter culture usage in food industries in developed countries involves the use of advance technology, process parameters under controlled conditions (Siragusa et al., 2009; Gänzle & Vogel, 2003; Buckenhüskes, 1993). The three types of controlled fermentation based on bioreactor designs are batch, fed-batch and continuous fermentation (Paulová et al., 2013; Stanbury et al., 2013). In controlled fermentation, the raw material/substrate is mostly sterilized using heat treatment or some other means; it is then inoculated with high population (about 10^6 cells /ml) of the selected single or mixed pure strains/starter cultures. Commercial starter cultures are available for such purposes. Optimum incubation conditions are then set for the growth and proliferation of the starter cultures. This method of fermentation produces large volumes of products with assured quality and consistency. Desirable secondary flora may be absent resulting in the nonproduction of certain desired flavours (Bibek, 2004). Some of the LAB species used in the preparation of commercial starter cultures include *Lactobacillus plantarum, L. sanfranciscensis, L. casei, L. fermentum, L. reuteri, L. helviticus, L. paracasei, L. brevis, L. delbrueckii, P. acidilactici*. The yeast species include *S. cerevisiae, Torulaspora delbruckii, Candida milleri* and *S. pastorianus*. They are mostly sold in a freeze dried, spray dried or frozen state (Brandt, 2014; Poitrenaud, 2003). Some starter microbial isolates from indigenous fermented African foods have been reported and used in trial experiments. *L. fermentum, L. brevis, L. plantarum, P. pentosaceus, S. cerevisieae* and *C. tropicalis* are a few. These were used either in single or in combinations (Annan et al., 2015;

Mugula et al., 2003b). Teniola and Odunfa (2001) reported the use of S. *cerevisiae* and *L.brevis* as starter cultures for the production of maize ogi (porridge) with an increase in the amino acids.

CHAPTER THREE

3.0 Bacterial diversity and metabolites profiling during *Hausa koko* production

3.1 Introduction

Increasing population and consumers demand for quality and safe fermented foods has given rise to several advances in fermentation technology research. One of such is the emergence of culture independent high-throughput sequencing methods and metabolomic analysis (Gao et al., 2021; Chen et al., 2017). Culture independent high-throughput sequencing methods for identifying microbial communities allows for their in-depth understanding, rather than the culture dependent methods that have limitation but help in the phenotypical testing of isolates. Sequencing based methods are more sensitive as they are able to identify microorganisms occurring in very low populations and those that otherwise would not have been isolated on growth media. Advent of the high-throughput methods (such as described in Section 2.5.4) have therefore uncovered many species than were known before in different fermented food communities (Bourrie et al., 2016; Dobson et al., 2011).

Metagenomics, also known as community genomics refer to high-throughput sequencing based analysis of the microorganisms in an environmental/community sample to identify their diversity and function. The environmental/community sample, which can be obtained as total microbial DNA containing all the genes in the sample is sequenced. Different sequencing approaches can successfully be applied to metagenomic studies with varying levels of reliabilities (Durazzi et al., 2021; Solieri et al., 2013; Jung et al., 2011; Rodriguez-Brito et al., 2006). One such high-

throughput sequencing approach for bacterial diversity profiling being used currently is 16S ribosomal ribonucleic acid (16S rRNA) gene amplicon sequencing on Next Generation Sequencing (NGS) platforms (Bourrie et al., 2016; Chaudhary et al., 2015; Dobson et al., 2011). This focuses on hypervariable regions instead of full-length gene sequencing for precise and reliable taxonomic classification at an affordable cost (Chaudhary et al., 2015). One of the disadvantages of using 16S rRNA however is its inability to provide the functional capabilities of the microbes and limitation on phylogenetic analysis (Peterson et al., 2021). In times past, despite the accuracy and depth of information culture–independent methods provide, the microbiota of only few fermented foods had been reported in Africa. This narrative is however changing. Diaz et al., (2019) reported the use of only V4 hyper variable regions analysed by 16S rRNA gene amplicon sequencing for several African fermented foods including *ogi, kwerionik, mawe, boule d'akassa, fura* and *dawadawa* to profile the microbiota for those foods. Similarly, Gabaza et al., (2019) used high throughput sequencing methods to identify the bacterial diversity of some cereals including millet, maize and sorghum used for preparation of porridge from different locations in Zimbabwe. Other fermented foods have also been reported (Ezekiel et al., 2019; Parker et al., 2018; Assohoun-Djeni et al., 2016).

Metabolomic analysis or metabolic profiling helps in identifying and quantifying intracellular metabolites which are low molecular compounds (< 1500 Da) produced by microorganisms during fermentation processes (Adebo et al., 2017; Mozzi et al., 2013; Weckwerth & Fiehn, 2002). These metabolites, including amino acids, organic acids, vitamins, minerals, polyphenols and many others can be profiled using detection tools such as Nuclear Magnetic Resonance (NMR) spectroscopy, Near Infrared spectrometry (NIR), Mass Spectrometry (MS) and others. With this information, it is possible to predict the nutritional quality of the fermented product. Out of these

detection methods, NMR can profile all compounds using NMR-measurable nuclei (Mozzi et al., 2013). It has been extensively used in advanced countries mostly to profile metabolite changes in fermented foods. For instance, changes in metabolits profile was reported during the fermentation of meju and contributed to their sensory qualities (Kang et al., 2011). It has been used to predict the sensory attributes of different wines (Rochfort et al., 2010). Information is however scarce when it comes to its application on African fermented foods.

The use of such advanced technologies to analyse the bacterial diversity and metabolites in African fermented foods will facilitate improvement and innovation in the industry. Whilst information is available on the lactic acid bacteria population of *Hausa koko*, a nutritious fermented millet porridge from Ghana (Lei & Jakobsen, 2004), there is no information regarding its bacterial community and the metabolites produced. The objectives of this study therefore were to determine the bacterial community at different stages of *Hausa koko* production using culture-independent high throughput sequencing of the 16S rRNA gene and metabolites produced. This will help to unveil the unreported bacteria communities and the metabolites produced for better understanding of the production dynamics during processing at the different stages.

3.2 Materials and Methods

3.2.1 Study design

Hausa koko production sites located in six regions of Ghana and spread through the northern, middle and southern agro-ecological belts were identified and selected for samples for the study. Samples of dry millet grains (raw materials), *Hausa koko* (finished product) and five intermediate products were collected during hausa koko production at the different sites, for various analyses.

3.2.2 Sampling sites and sampling

The selected locations were:

Northern Region: Tamale Central (TAC), Tamale Kalariga (TAK), Tamale Dabokpa (TAD); Bono East Region: Techiman Diasempa (TED), Techiman Abourso (TEA), Techiman Pomaakrom (TEP); Bono Region: Sunyani (SUN); Central Region: Mankessim (MAN); Eastern Region: Dodowa (DOD); Greater Accra Region Accra: Ashaiman-Tulaku (AAT), Accra Madina Zongo (AMZ), Accra Ashaley Botwe (AAB).

The samples collected during processing at each of the sampling sites were; Dry millet grains (D); 12 h steeped millet (12 h); 24 h steeped millet (24 h); Milled millet with spices (M); Supernatant of fermented slurry (Su); Sediment of fermented slurry (Sd) and *Hausa koko* (K).

Duplicate samples (500 g each) were collected aseptically into sterile sampling containers and transported to the Microbiology laboratory at CSIR-Food Research Institute (FRI) in Accra under cold storage and preserved at at -20 °C. They were later transported to Quadram Institute

Biosciences (QIB, UK) under cold storage condition with frozen ice packs and preserved at -20 °C for analysis.

3.2.3 Sample analyses

3.2.3.1 pH measurement

The pH of liquid samples (20 ml) were taken directly after homogenization whilst solid samples (10 g) were homogenised with 20 ml of sterile ultrapure water and determined using pH meter (MettlerToledo, Switzerland) after calibration with standard buffers.

3.2.3.2 Total microbial DNA extraction from fermented samples

The entire method is as described by Atter et al., (2021) and Diaz et al., (2019). This protocol includes a first step in which the microorganisms are separated from solid particles and a second step involving the extraction of the microbial DNA using the FastDNA spin kit. For the first step, fermented samples at the different stages of processing were thawed on ice. Twenty grams (20 g) were transferred into a sterile 50 ml screw cap tube and 10 ml ice cold ultrapure water was added, homogenized by vortexing and centrifuged (Eppendorf 5810R, Germany) for 1min at 800 g at 4 °C to remove the solid particles of the sample. This ratio of sample (20 g): ultrapure water (10 ml) was used only for the supernatants whilst for the other samples, the ratios were modified to 20 g dry grains: 30 ml water, 10 g milled millet: 20 ml water and 20 g sample (12 h, 24 h, sediments and *koko*): 20 ml water. The ratios were varied to obtain maximum extract from the samples. The supernatants, which contained the microorganisms, were transferred to new 50 ml tubes. These were repeated twice and the supernatants per sample pooled together into one tube with a final volume of about 30 ml. Supernatants were centrifuged at 3000 x *g* at 4°C for 20 min to pellet the cells and supernatants were discarded. The pellets were washed by re-suspending in 1 ml Phosphate Buffered Saline (PBS) buffer, transferred to 2 ml screw cap tubes and centrifuged at

14000 x *g* for 2 min and two more washes were performed using PBS buffer. In the second step, FastDNA spin kit for soil (MP Biomedicals, USA) was used. The pellet was resuspended in 978 µl Sodium Phosphate Buffer and 122 µl MT buffer, vortexed, incubated in the refrigerator (4 °C) for 1 h whilst vortexing to homogenise every 15 min. The sample (1 ml) was transferred into a Lysis Matrix E Tube, the cap tightened up and homogenized using FastPrep-24 instrument (MP Biomedicals, UK) for 60 s at a speed of 6.5 m/s. The FastPrep homoginization was repeated three times with the samples kept on ice for 5 min for each homogenization break. Lysing Matrix E tubes were centrifuged for 1 min at 16,800 x *g*. Supernatant was transferred into a clean Eppendorf tube, 250 µl PPS reagent was added, mixed by shaking and inverting the tube vigorously by hand 10 times, and centrifuged for 5 min at 16800 x *g* to pelletize. Supernatant was then transferred into a sterile 15 ml tube, 1 ml of Binding Matrix Suspension was added, tubes were inverted by hand for 2 mins after which the tubes were incubated in a rack for 3 mins to allow for the settling of silica matrix. One ml of supernatant was removed and the remaining re-suspended. The mixture (600 µl) was transferred into a SPIN filter tube and centrifuged for 1 min at 14,500 x *g*. The remaining mixture was added and centrifuged as well. The flow-through was decanted and 500 µl of SEWS-M wash solution was added into the SPIN filter tube and centrifuged for 1 min at 14,500 x *g*. The flow-through washing was decanted and repeated two more times. The flow-through was decanted and centrifuged for an additional 2 mins at 14,500 x *g* to dry matrix of residual SEWS-M wash solution. And spin filter was removed and placed in a fresh Catch Tube, prior to air-drying for 5 mins at room temperature. DES (DNase/Pyrogen free water) was warmed at 55 °C for 5 mins after which 50 µl was added to the matrix of the air-dried spin filter, incubated at room temperature for 1 min and centrifuged for 1 min at 14,500 x *g* to elute DNA. DNA samples were stored at -20 °C until used for further assays.

3.2.3.3 DNA quantification

DNA concentrations were measured with the Qubit 3.0 fluorometer (Invitrogen, Malaysia) using the Qubit dsDNA Broad Range (BR) Assay kit (Invitrogen) or the Qubit dsDNA High Sensitivity (HS) Assay kit (Invitrogen). The BR quantifies between 0-100 ng/µl whilst the HS quantifies between 0-10 ng/µl of DNA in a sample (Atter et al, 2021; Wong, 2018).

3.2.3.4 16S rRNA gene amplicon sequencing analysis

Bacterial diversity was analysed by 16S rRNA gene amplicon high throughput amplicon sequencing of the total microbial DNA. Novogene Co., Ltd (Hong Kong) carried out the amplification and sequencing as follows. The V4 hypervariable region of the 16S rRNA gene was amplified by PCR using specific primer pair 515 F (GTGCCAGCMGCCGCGGTAA) and 806 R (TAATCTWTGGGVHCATCAGG) (Caporaso et al., 2010) and the Phusion High-Fidelity PCR master mix (New England Biolabs, England), following manufacturer´s instructions. The amplicons were used to generate libraries using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, England) and then sequenced using paired-end Illumina sequencing (2 × 250 bp) on the HiSeq 2500 platform (Illumina, USA). Taxonomic assignment of bacteria at 97 % similarity was performed using Naïve Bayes classifier trained on the Silva version 138.99 % operational taxonomic unit (OTU) database. The pair-wise comparisons of alpha diversity was performed in Quantitative Insights Into Microbial Ecology 2 (QIIME2) v. 2019.7 software. Statistical analysis in beta diversity was performed with permanova method and pseudo-F test. Venn diagram was constructed using https://bioinfogp.cnb.csic.es/tools/venny/ software.

3.2.3.5 Metabolomics analysis

The metabolomics analysis method used on samples (D, 12 h, 24 h, M, Su and K) are as reported by Atter et. al., (2021). Samples extracted with NMR buffer (4.2 g $NaH_2PO_4.H_2O$, 3.3 g K_2HPO_4, 17.2 mg of Na_3PO_4, 20 mg NaN_3 in 100 µl × 100 mM EDTA in H_2O) were run on 600 MHz Bruker NMR with cryoprobe. Metabolites were identified and quantified by computer-assisted manual fitting with Chenomx NMR suite v 8.12 (Chenomx, Edmonton, Canada), using Chenomx 600 MHz HMDB Compounds library. Statistical analysis was conducted using two-way ANOVA with Tukey's multiple comparisons test where at p-value ≤ 0.05, a significant difference was applied using GraphPad prism v8.4.3.

3.3 Results

3.3.1 pH of samples at different stages of fura production

The pH of the samples from the different processors varied along the processing stages (Figure 5a). The pH of the dried millet samples ranged from 5.45-6.58 but reduced drastically to 3.51-3.99 in the *Hausa koko* samples.

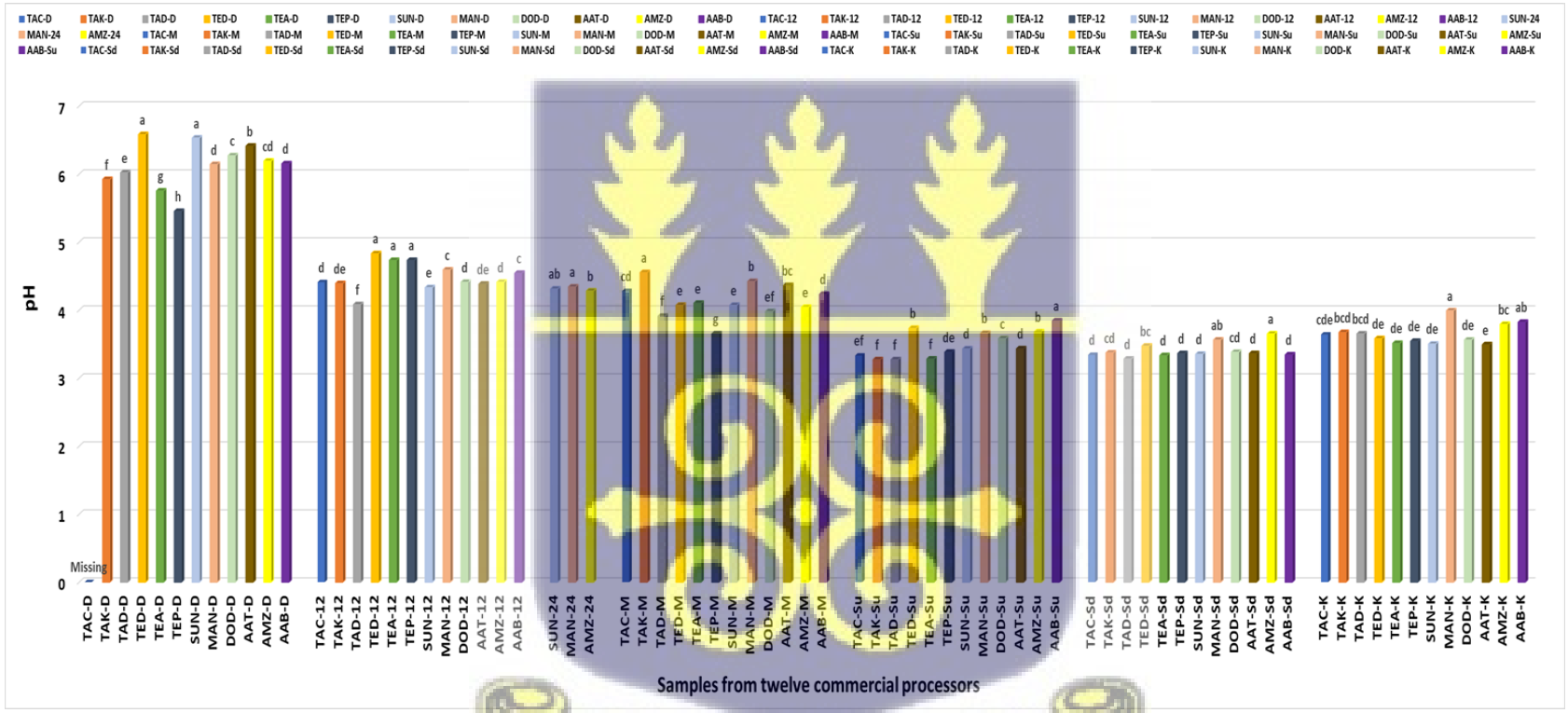


Figure 5a. pH values of samples at different processing stages from 12 commercial processors

NB:

a - f = bars with different letters are significantly different at $P \leq 0.05$

Sampling points:

D = dry millet grains; 12 = 12 h steeped millet; 24 = 24 h steeped millet; M = milled millet with spices; Su = supernatant of fermented slurry; Sd = sediment of fermented slurry; K = *Hausa koko*

Sampling sites:

Tamale Central (TAC); Tamale Kalariga (TAK); Tamale Dabokpa (TAD); Techiman Diasempa (TED); Techiman Abourso (TEA); Techiman Pomaakrom (TEP); Sunyani (SUN); Mankessim (MAN); Dodowa (DOD); Accra Ashaiman-Tulaku (AAT); Accra Madina Zongo (AMZ); Accra Asharley Botwe (AAB)

3.3.2 Bacterial diversity analysed by 16S rRNA amplicon sequencing

Analysis of the V4 hypervariable region of the 16S rRNA gene amplicon using high throughput amplicon sequencing generated over 8,000,000 paired-end sequence reads. Some of these reads identified as chimeras and those deemed as poor quality were discarded. Sequences assigned as chloroplast and mitochondrial were also removed. The generated rarefaction curves (calculation of species richness) constructed for the operational taxonomic units (OTUs) totalling 49,824 were adequate to capture most of the microbial diversity in all the samples from the various stages of the production process as shown in Figure 5b.

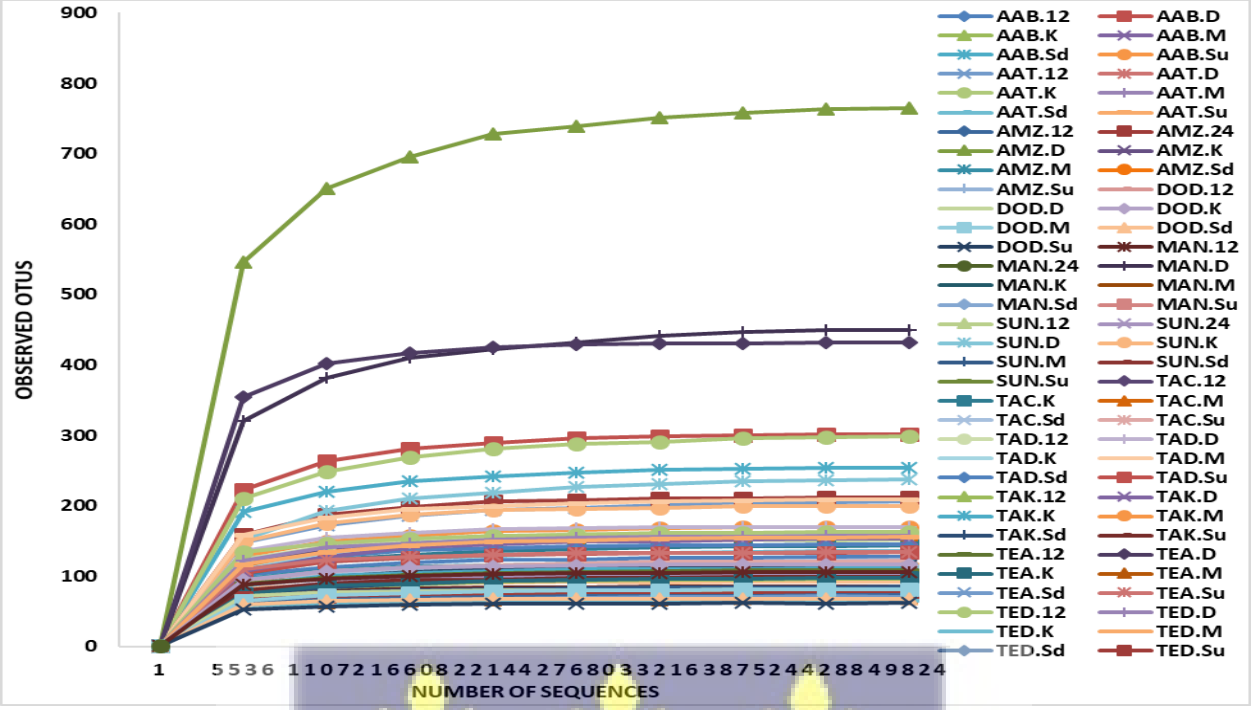


Figure 5b. Rarefaction curves of OTUs to a depth of 49824

NB:

Sampling points:

D = dry millet grains; 12 = 12 h steeped millet; 24 = 24 h steeped millet; M = milled millet with spices; Su = supernatant of fermented slurry; Sd = sediment of fermented slurry; K = *Hausa koko*

Sampling sites:

Tamale Central (TAC); Tamale Kalariga (TAK); Tamale Dabokpa (TAD); Techiman Diasempa (TED); Techiman Abourso (TEA); Techiman Pomaakrom (TEP); Sunyani (SUN); Mankessim (MAN); Dodowa (DOD); Accra Ashaiman-Tulaku (AAT); Accra Madina Zongo (AMZ); Accra Asharley Botwe (AAB)

Gram-positive and Gram-negative bacteria were identified at the various stages of production of *Hausa koko* from all the twelve different processors. The composition of the bacteria communities compared at the different taxonomic levels showed that the dominant OTUs in the samples were from the kingdom (Bacteria); phylum (Firmicutes, Proteobacteria); class (Bacilli, Alphaproteobacteria, Gammaproteobacteria); order (*Lactobacillales, Rhodospirillales, Enterobacteriales*); family (*Lactobacillaceae, Leuconostocaceae, Streptococcaceae, Acetobacteraceae, Enterobacteriaceae*); genus (*Lactobacillus, Pediococcus, Leuconostoc, Weissella, Lactococcus, Streptococcus, Acetobacter, Gluconobacter, Pantoea*). More than four hundred (400) different Gram positive and negative bacteria were identified at the different stages from the different processors. The bacterial population was more diverse in the dry grains than in the intermediate and finished products. The grain samples were dominated by potential pathogenic bacteria including Enterobacteriaceae, *Staphylococcus, Escherichia-Shigella* among others but their population reduced along all the fermentation stages (12 h, 24 h, M, Su and Sd). Generally, the bacterial community found at the same stages of the production process was similar across samples from the twelve processors irrespective of the locality. Some bacteria were also specific to stages of production. However, significant differences in the relative abundance depending on the processing step was observed. The OTUs with relative abundance higher than 0.01 % in at least one sample are presented in Figure 6a.

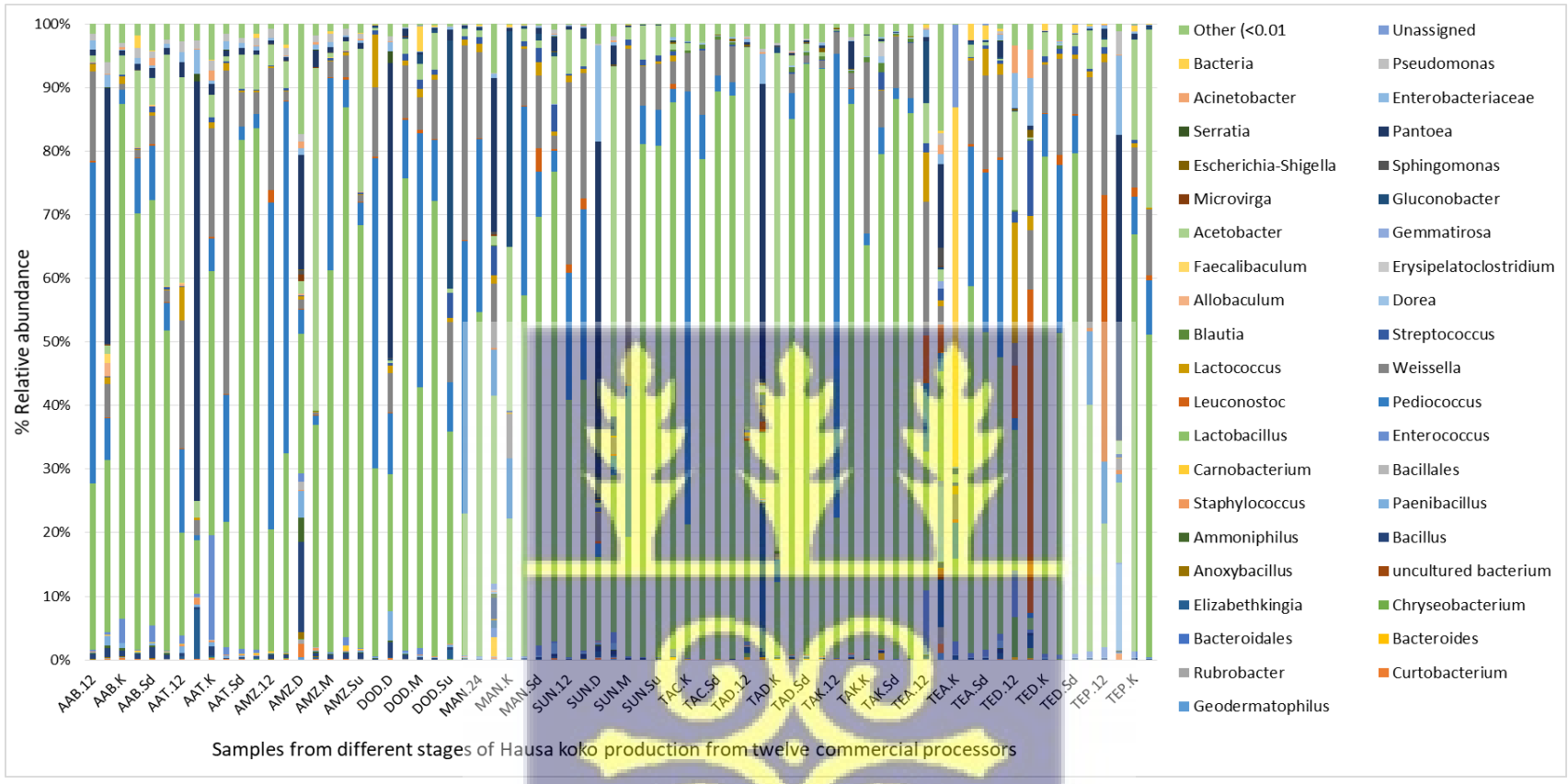


Figure 6a: Relative abundance of the operational taxonomic units (OTUs) among the twelve processors

NB:

Sampling points:

D = dry millet grains; 12 = 12 h steeped millet; 24 = 24 h steeped millet; M = milled millet with spices; Su = supernatant of fermented slurry; Sd = sediment of fermented slurry; K = *Hausa koko*

Sampling sites:

Tamale Central (TAC); Tamale Kalariga (TAK); Tamale Dabokpa (TAD); Techiman Diasempa (TED); Techiman Abourso (TEA); Techiman Pomaakrom (TEP); Sunyani (SUN); Mankessim (MAN); Dodowa (DOD); Accra Ashaiman-Tulaku (AAT); Accra Madina Zongo (AMZ); Accra Asharley Botwe (AAB)

The relative abundance of the top 20 OTUs at the genus level for grouped samples from the different processors across the six regions is presented in Figure 6b. The bacterial microbiota at the different levels indicated varying increasing or decreasing percentages depending on their importance and specific roles being played. For instance, the bacterial population was dominated by the genus *Pantoea* in the grain samples, but their population decreased in the fermentation stages. These fermentation stages were mainly dominated by *Lactobacillus* with others such as *Pediococcus*, *Weissella*, *Acetobacter*, *Leucnostoc*, *Lactococcus* occurring in low populations.

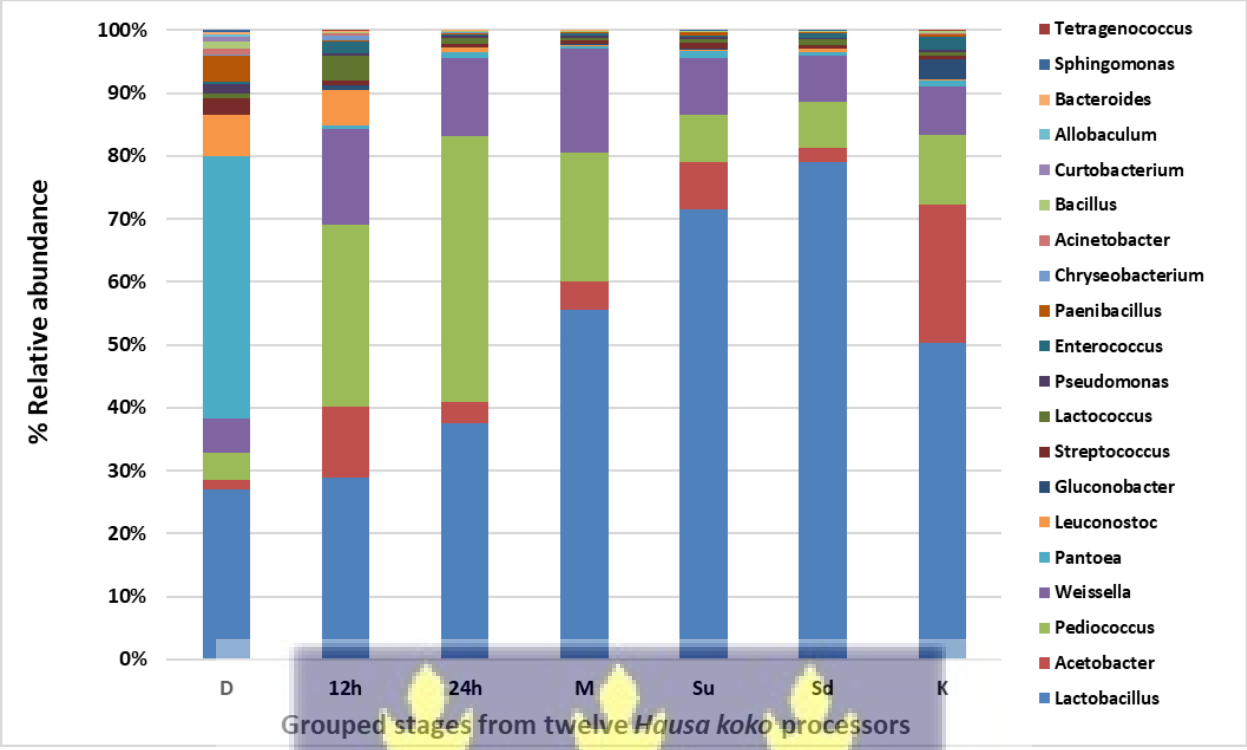


Figure 6b: Relative abundance of the top 20 abundant operational taxonomic units (OTUs) at the genus level of the different processing stages from the twelve processors

NB:

Sampling points:

D = dry millet grains; **12h** = 12 h steeped millet; **24h** = 24 h steeped millet; **M** = milled millet with spices; **Su** = supernatant of fermented slurry; **Sd** = sediment of fermented slurry; **K** = *Hausa koko*

Venn diagrams showing the shared operational taxonomic units between the different stages during *Hausa koko* production from twelve producers are shown in Figure 7. Each petal in the Venn diagram indicated by a colour represented one group of samples at a production stage. The numbers on the petals representing the unique OTU number or cluster of closely related variants of the gene sequence of each stage sample and how they were interrelated to other stages are shown

(Figure 7). The overlapping numbers represents the common OTUs of the grouped stage samples. A comparison of the OTUs shared by the first set of four different grouped stages (D, 12 h, 24 h and M) revealed that the highest number of OTUs between a pair were shared between D and 12 h with 39 OTUs translating into 5.3 %. The second set of four different grouped time points (M, Su, Sd and K) revealed stages Sd and K shared the most (27) OTUs forming 5.6 % of total OTUs with Su and K sharing the least (11) OTUs representing 2.3 %. The entire first set shared 85 OTUs representing 11.6 % whilst the entire second set shared 135 OTUs (27.9 %).

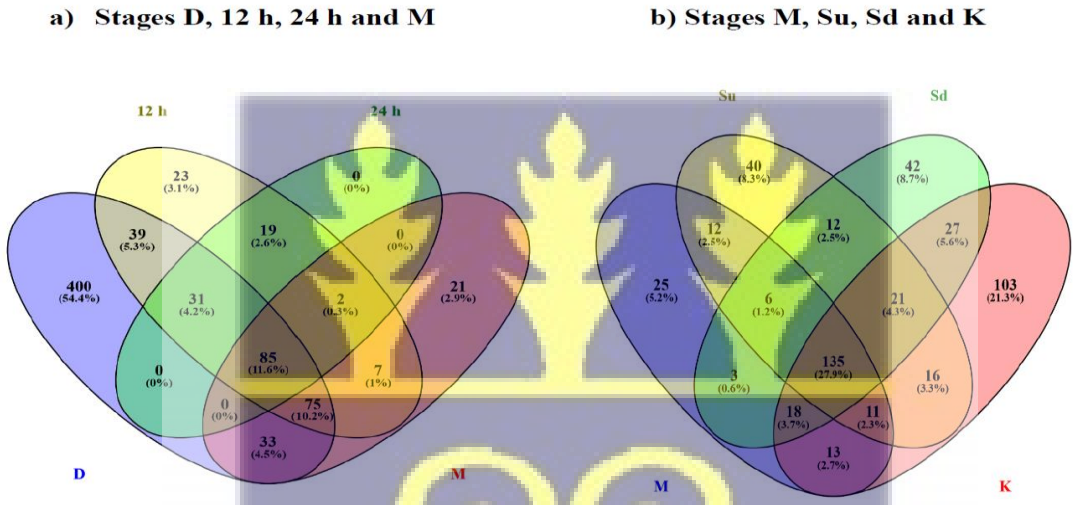


Figure 7: Venn diagram showing the shared operational taxonomic units between the different stages during *Hausa koko* production from twelve producers a) D, 12 h, 24 h and M b) M, Su, Sd and K.

NB:

Sampling points:

D = dry millet grains; 12 = 12 h steeped millet; 24 = 24 h steeped millet; M = milled millet with spices; Su = supernatant of fermented slurry; Sd = sediment of fermented slurry; K = *Hausa koko*

Weighted UniFrac distance was used in comparing the populations between the different stages with respect to the relative abundance of the taxa. The results from the pairwise comparisons of alpha diversity performed in qiime2 v. 2019.7 showed that the observed OTUs in grain samples were higher than in the rest of the other time points (p-value = 0.009). Outliers (grey dots) were observed only in grain, sediment and *koko* samples as shown in Figure 8a.

Comparing the same processing stage between samples from the six regions showed some significant differences ($P \leq 0.05$) amongst a few of them using kruskal-wallis pairwise comparisons of alpha diversity. For instance, these differences were found in *koko* samples between Accra and Techiman (higher in Accra) and between Tamale and Techiman (higher in Tamale). In sediment samples the observed OTUs were lower in Techiman samples than in Accra and Tamale (Figure 8b). There were no significant differences between other samples within same stages from the different regions. Significant differences also existed between different processing stages within a region. For instance, there were significance differences (p-value 0.049535) in Techiman samples where the observed OTUs were higher in grains than sediment, 12 h than sediment, sediment than supernatant and grains than *koko*. Sediment samples from Bono East were also significantly different (p-value 0.033895) from sediment samples from Greater Accra and Northern Regions whilst those from Eastern and Central Regions were not.

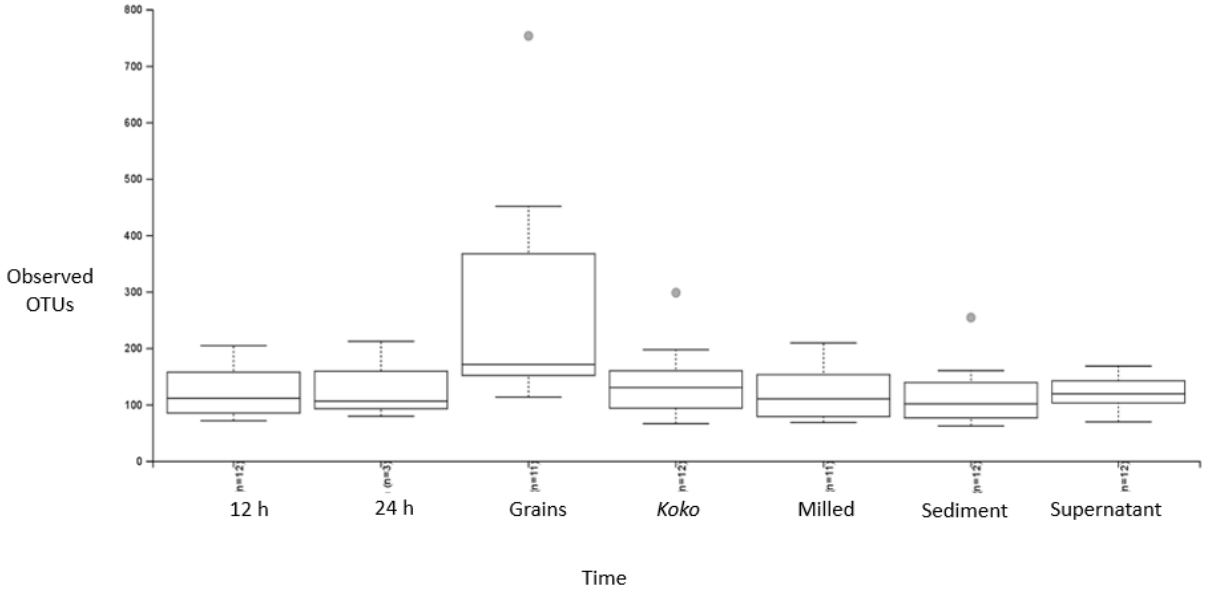


Figure 8a: Observed OTUs based on time points (Alpha diversity)

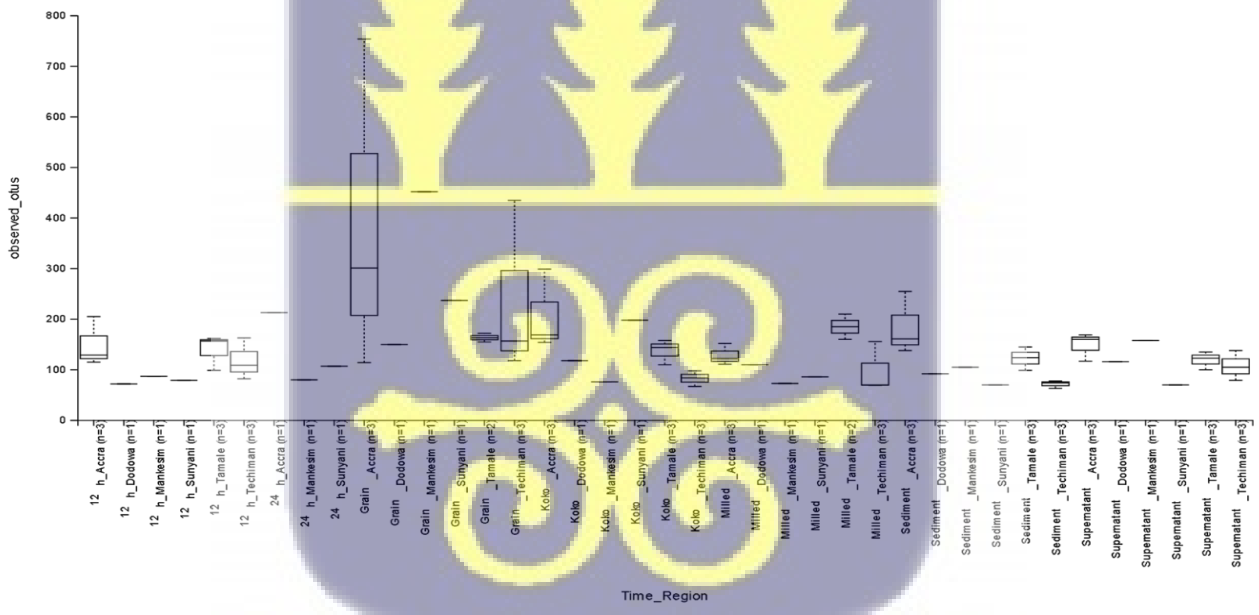


Figure 8b: Comparative diversity of microbes within fermentation stages/time points between regions (Alpha diversity)

NB:

Sampling points:

D = dry millet grains; 12 = 12 h steeped millet; 24 = 24 h steeped millet; M = milled millet with spices; Su = supernatant of fermented slurry; Sd = sediment of fermented slurry; K = *Hausa koko*

Significant differences (p-value = 0.024) were again found between the different processing stages using unweighted unifrac distances. Grain samples for instance were significantly different than all the other processing stages (p-value grain vs 12h = 0.002, grain vs koko = 0.031, grain vs milled = 0.007, grain vs sediment = 0.004, grain vs supernatant = 0.002). The data also showed that the microbial population were highly diverse among the grain samples. The separation of grain samples was mostly caused by OTUs within the Gammaproteobacteria class (Figure 9a). There were also significant differences (p-value=0.001) based on the regional comparism.

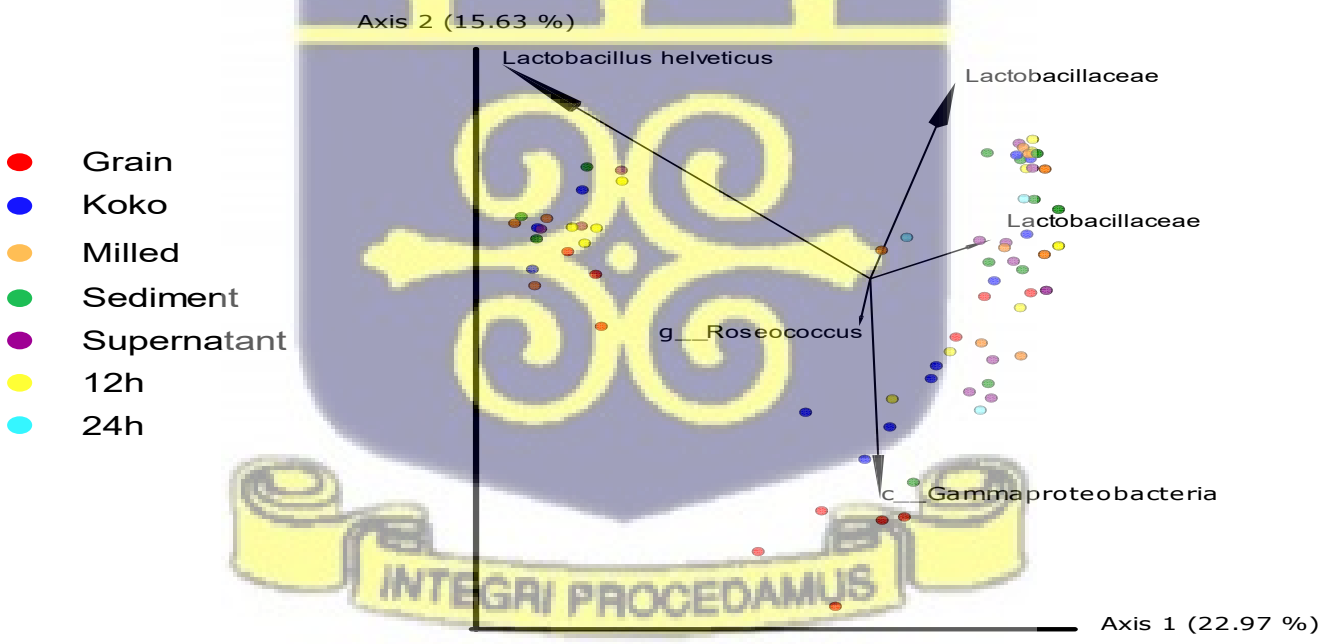


Figure 9a: PCoA biplot based on unweighted unifrac distance showing the distribution of the samples based on the different processing stages (Beta diversity)

Significant differences (p-value = 0.001) were found between samples produced from the Northern Region and the rest of the other Regions as shown (Figure 9b). The significant differences between samples from the Northern Region and the rest of the samples from other regions was mostly caused by OTUs classified as *L. helveticus* (Figure 9c).

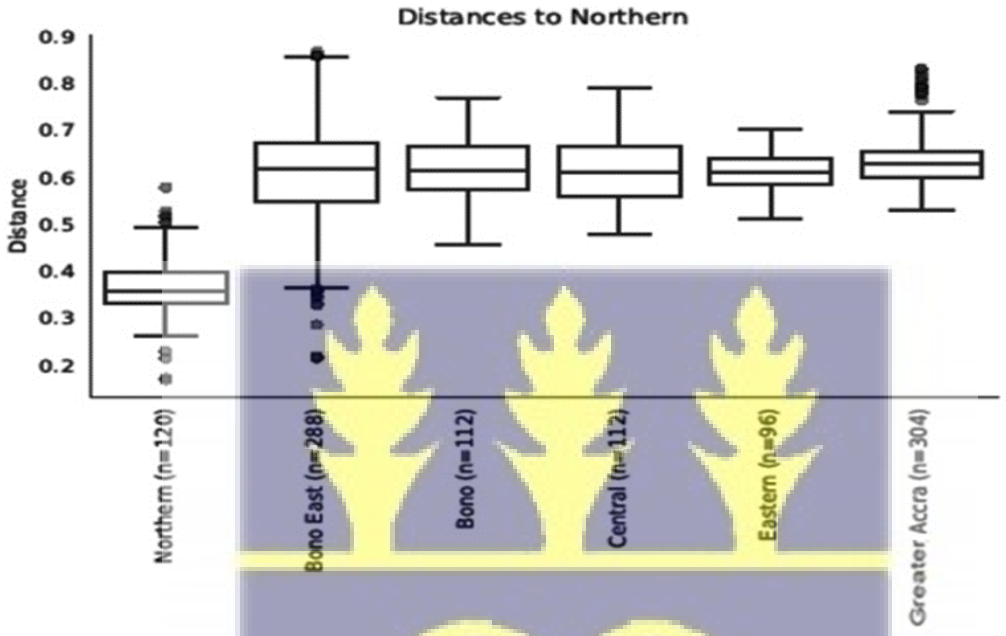


Figure 9b: Boxplot of Unweighted unifrac distances showing how distant other regional samples were to the Northern Region samples (Beta diversity)

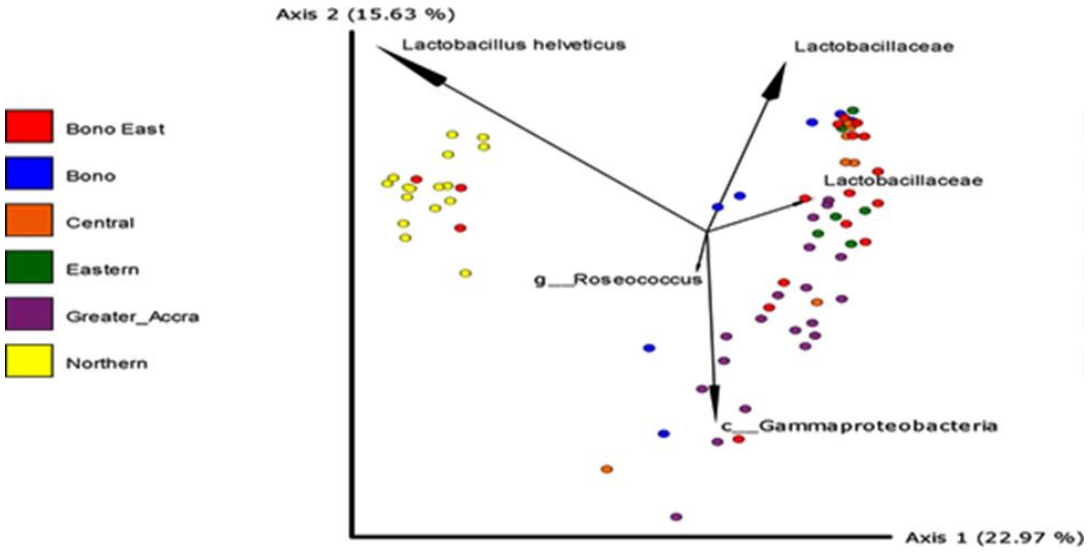


Figure 9c: PCoA biplot based on unweighted unifrac distance showing the distribution of the samples based on the different Regions (Beta diversity)

3.3.3 Metabolites produced

The selected *Hausa koko* production stages (D, 12 h, 24 h, M, Su and K) from twelve producers detected wide varieties of metabolites identified using Chenomx NMR suite v 8.12. A total of 33 different metabolites were identified. They include organic compounds, alcohols, sugars, amino acids, with intermediary compounds and some other key metabolites. Their concentrations varied in similar patterns with increasing and decreasing trends in all samples along the different production stages irrespective of the geographical location they were obtained from. Smaller quantities and numbers of metabolites were obtained from the grain samples as compared to the rest of the processing stages.

Considering samples between and within regions for instance, even though similar metabolites were produced, most of their mean concentrations were significantly different (p-value=0.0002). For instance, comparing the concentrations of the amino acid, valine, for samples TED (from

Techiman) and AAB (from Accra), these differences were obvious at some of the stages. TED-D (0.0291 mmol/100g), AAB-D (0.0974 mmol/100g); TED-12 (0.1288 mmol/100g), AAB-12 (0.6538 mmol/100g); TED-M (0.2057 mmol/100g), AAB-M (0.2862 mmol/100g); TED-Su (0.0131 mmol/100g), AAB-Su (0.04964 mmol/100g); TED-K (0.00172 mmol/100g), AAB-K (0.01168 mmol/100g). Again, within regions, these differences existed. For instance, the mean lactate concentrations between the two supernatant samples from Tamale, TAC and TAK, varied as TAC-Su, 7.54868 mmol/100g) and TAK-Su 1.7179 mmol/100g., Ethanol concentration also varied as: TAC-Su 4.31866 mmol/100g and, TAK-Su 0.9423 mmol/100g. For succinate, the concentrations were: TAC-Su 0.12148 mmol/100g and TAK-Su 0.0006 mmol/100g. There were certain metabolites such as acetate, alanine, betaine, choline, ethanol, formate, glycine, succinate and valine that were present in varying concentrations in all the samples at the different processing stages. Increasing progression of organic compounds was generally observed. Lactate concentration for instance increased from a range of 0.0000 - 0.6916 mmol/100g in grain samples to 2.4209 - 8.8236 mmol/100g in 12 h fermented millet; and 6.3618 - 7.8869 mmol/100g in 24 h, 0.1640 - 12.6338 mmol/100g in milled samples containing spices, 1.24948 - 7.54868 mmol/100g in supernatants, and 0.49882 - 2.69954 mmol/100g in *Hausa koko*. Generally, there was a reduction in the sugar concentrations despite some few inconsistencies. There were drastic increments in sugar concentrations in all the milled samples. For instance, glucose, the most abundant sugar in the samples, recorded concentrations that ranged from 1.3168 - 9.0188 mmol/100g in grain samples, 0.0000 - 5.3986 mmol/100g in 12 h and 2.6560 - 4.9910 mmol/100g in 24 h. The concentrations were higher in the milled samples, ranging from 0.5514 to 19.6276 mmol/100g, 0.0000 - 0.0493 mmol/100g in Su and 0.0000 - 0.64606 mmol/100g in *Hausa koko* samples. The other sugars were observed at very low concentrations.

The concentration of amino acids mostly increased significantly ($p < 0.0001$) from the grains to the 12 and 24 fermentation and finally to the milled samples. They however reduced in the supernatant and *Hausa koko* samples. The mean concentration of the metabolites produced (33) at three processing stages (D, M and K) from the commercial processors are shown in Table 1a, b, and c.

Table 1a: The mean concentration (mmol/100 g) of metabolites produced in Dry millet (D), samples from commercial processors

Metabolite	TAC-D	TAK-D	TAD-D	TED-D	TEA-D	TEP-D	SUN-D	MAN-D	DOD-D	AAT-D	AMZ-D	AAB-D
Acetone	(Missing)	0.147	0.0662	0.0798	0.0815	0.151	0.139	0.0544	0.072	0.1273	0.1553	0.0805
Acetoin		0	0	0	0	0	0	0	0	0	0	0
Ethanol		0.8738	0.9550	0.0967	1.0867	0.9669	0.5717	0.6031	0.9202	0.7013	1.0668	0.9356
Acetate		0.2322	0.1198	0.2442	0.0834	0.2489	0.1422	0.1910	0.1318	0.1458	0.1329	0.1501
Formate		0.0441	0.0578	0.0957	0.0619	0.0577	0.023	0.0417	0.0518	0.0362	0.0864	0.0618
Lactate		0.6916	0	0.0421	0	0.0516	0.0901	0.0913	0.1005	0.1415	0.0552	0.0731
Asparagine		0.4864	0.4601	0.2295	0.4093	0.4633	0.4036	0.4985	0.5054	0.5206	0.4569	0.5163
Glycine		0.5134	0.1686	0.0714	0.1573	0.4849	0.6304	0.3410	0.1738	0.1226	0.3953	0.8928
Leucine		0.1333	0.0631	0.0375	0.0678	0.1594	0.0913	0.1103	0.0867	0.1218	0.1002	0.0824
Tyrosine		0.0781	0.0555	0.0279	0.0532	0.0792	0.0492	0.0636	0.053	0.0632	0.0686	0.0615
Isoleucine		0.0821	0.0437	0.023	0.032	0.0911	0.0481	0.0612	0.0475	0.085	0.1078	0.0538
Aspartate		0	0	0.0637	0	0	0	0	0	0	0	0
Glutamate		0	0	0	0.3011	0.4655	0.193	0.2203	0.2549	0.2791	0	0
Glutamine		0	0	0	0	0	0	0	0	0	0	0
Alanine		0.2391	0.1905	0.0865	0.1078	0.3145	0.2015	0.2488	0.1552	0.2731	0.1981	0.174
Threonine		0	0	0	0	0	0	0	0	0	0	0
4-Aminobutyrate		0.3057	0.1528	0.0882	0.1644	0.227	0.1802	0.1932	0.1682	0.2474	0.2166	0.1836
Valine		0.1309	0.0824	0.0291	0.0644	0.1629	0.0964	0.1131	0.0905	0.1214	0.1021	0.0974
Methionine		0.0640	0.0311	0.0173	0.025	0	0	0	0	0	0.0401	0.0387
Tryptophan		0	0	0.0136	0	0.0296	0.0159	0.0121	0.0213	0.026	0.023	0.0228
Phenylalanine		0.0609	0	0	0	0	0	0.0501	0.0442	0.06	0.0508	0.0425
Fructose		3.8429	3.8108	1.3539	3.5443	4.6915	1.1331	3.7237	1.748	3.8379	3.9815	4.4016
Sucrose		0.6552	0.6112	0.9512	0.824	0.3649	0.2317	0.3101	0.229	0.3081	0.313	0.4734
Galactose		0.4747	0.4139	0.2328	0.4748	0.572	0.3508	0.4254	0.4398	0.4447	0.4504	0.5668
Arabinose		0	0	0	0	0	0	0	0	0	0	0
Mannose		0.1121	0	0	0	0	0	0	0	0	0	0
Glucose		5.963	7.5329	1.3168	5.7879	6.3794	6.5926	6.4518	4.9909	9.0188	7.7264	6.8547
Maltose		0	0	0	0	0	0	0	0	0	0	0
Betaine		0.167	0.2089	0.0957	0.2331	0.2668	0.1769	0.1443	0.3072	0.155	0.2949	0.2158
Trigonelline		0.0377	0.033	0.0193	0.033	0.0376	0.0276	0.0342	0.0319	0.0298	0.0388	0.0398
Uracil		0.0455	0.017	0	0	0	0	0	0	0	0	0
Choline		0.4083	0.3217	0.1246	0.3007	0.3633	0.2902	0.2912	0.3085	0.3388	0.3274	0.3726
Succinate		0.0497	0.0377	0.0166	0.0438	0.0747	0.0449	0.0396	0.0489	0.05	0.0377	0.0467

Table 1b: The mean concentration (mmol/100 g) of metabolites produced in Milled millet containing spices (M) samples from commercial processors processors

Metabolites	TAC-M	TAK-M	TAD-M	TED-M	TEA-M	TEP-M	SUN-M	MAN-M	DOD-M	AAT-M	AMZ-M	AAB-M
Acetone	0.0235	0.0912	0.0384	0.0614	0.0977	0.0395	0.0442	0.0941	0.0281	0.0868	0.1196	0.0651
Acetoin	0.0093	0	0.0865	0.0411	2.4312	0.2088	0.0296	1.0921	1.266	0.2686	2.2327	0.0494
Ethanol	5.7858	1.1635	16.623	0.2245	0.4182	4.4003	3.2144	3.1365	3.4473	2.2143	2.3653	23.0054
Acetate	1.2282	0.2373	0.9251	6.541	22.0748	10.3934	7.0263	1.8468	4.0736	6.1017	2.5519	3.2191
Formate	0.0338	0.0417	0.0261	0.0571	0.0748	0.1575	0.2656	0.0853	0.1037	0.2003	0.1029	0.0153
Lactate	1.9076	0.164	10.4612	7.2797	4.72	12.6338	6.0859	5.2192	8.9601	3.2124	7.2919	5.9265
Asparagine	0.1298	0.6027	0.1074	0.049	0	0.171	0.0733	0	0.2014	0.0571	0.1226	0
Glycine	0.2113	0.8477	0.6618	0.566	0.2202	0.2331	0.6323	0.5193	0.2805	0.333	0.1905	0.4407
Leucine	0.1178	0.1262	0.6945	0.2192	0.3269	0.4907	0.2407	0.4616	0.6192	0.2468	0.5575	0.3887
Tyrosine	0.0801	0.0788	0.3254	0.0629	0.1042	0.1353	0.0501	0.1288	0.1791	0.0426	0.1864	0.1629
Isoleucine	0.0757	0.0709	0.2681	0.0869	0.1505	0.1718	0.1034	0.1796	0.2024	0.1167	0.1829	0.1244
Aspartate	0.1831	0	0.4271	0.2419	0.1238	0.3262	0.2286	0.249	0.2773	0.231	0.2016	0.067
Glutamate	0	0	0.6827	0.3497	0	0.4772	0.2141	0.3428	0.5209	0.2093	0	0.4197
Glutamine	0.0877	0	0.2079	0	0	0	0	0.0735	0.0991	0	0	0.118
Alanine	0.3602	0.2286	1.3762	0.5588	1.1189	0.9611	0.4729	0.8028	1.0072	0.4433	0.7326	1.2013
Threonine	0.1676	0.1994	0.3286	0.2806	0.3178	0.231	0.1859	0.2245	0.3679	0.2447	0.2134	0.3135
4-Aminobutyrate	0.2748	0.1624	0.3507	0.1636	0.3635	0.3614	0.3464	0.5058	0.522	0.337	0.5389	0.3272
Valine	0.1352	0.1272	0.5618	0.2057	0.381	0.3356	0.2248	0.3425	0.4279	0.2091	0.361	0.2862
Methionine	0.0351	0.0501	0.233	0.0571	0.114	0.1323	0.068	0.0922	0.1128	0.047	0.1001	0.1134
Tryptophan	0.0193	0.0194	0.0481	0.0188	0.0278	0.0355	0.0211	0.0309	0.0396	0.0177	0.0285	0.0317
Phenylalanine	0.0742	0.0655	0.3296	0.10008	0.2062	0.2035	0.1181	0.183	0.225	0.1094	0.2205	0.1533
Fructose	0.5865	4.0397	0	0	0.8558	0	1.3061	0.7339	0.3635	1.5694	0	0
Sucrose	0	0	0	0	0	0	0	0	0	0	0	0
Galactose	0.322	0.6397	0	0.4604	0.5054	0.4603	0.7339	0.8717	0.6219	0.668	0.7994	0.5215
Arabinose	0	0	0	0	0	0	0	0	0	0	0	0
Mannose	0	0.0967	0	0	0	0	0	0	0	0	0	0
Glucose	1.9154	9.2578	0.5514	4.496	13.0801	5.0393	15.6187	8.3206	14.1374	19.6276	4.4825	1.2274
Maltose	0.117	5.4163	0	0	0	0	0.0703	0	0.0788	0.1181	0	0
Betaine	0.0625	0.2468	0.0822	0.1361	0.093	0.1738	0.109	0.091	0.18	0.0858	0.0759	0.0378
Trigonelline	0.0144	0.0482	0.0138	0.0128	0.0204	0.0182	0.0149	0.0182	0.0165	0.0149	0.0186	0.0176
Uracil	0.0659	0.0446	0.111	0.1103	0.1053	0.0683	0.0793	0.12	0.115	0.051	0.0979	0.086
Choline	0.1951	0.4893	0.3556	0.2756	0.3569	0.3427	0.2956	0.3236	0.3456	0.2726	0.3349	0.2888
Succinate	0.069	0.0431	0.1197	0.0513	0.0606	0.1757	0.0283	0.0578	0.0743	0.0171	0.0666	0.1417

Table 1c: The mean concentration (mmol/100 g) of metabolites produced in *Hausa* koko (K) samples from commercial processors

Metabolite	TAC-K	TAK-K	TAD-K	TED-K	TEA-K	TEP-K	SUN-K	MAN-K	DOD-K	AAT-K	AMZ-K	AAB-K
Acetone	0.1604	0.01818	0.01642	0.00792	0.02192	0.02226	0.0095	0.0454	0.00874	0.04484	0.01846	0.01912
Acetoin	0.0062	0	0	0	0	0	0.00706	0	0	0	0.00478	0
Ethanol	4.90332	4.81686	3.46462	1.65286	2.35746	0.34028	0.3332	0.28542	0.44534	0.43674	0.07576	0.22278
Acetate	0.40606	0.47866	0.21874	0.40002	0.29036	0.21986	0.24846	0.16654	0.21872	0.3124	0.26154	0.1269
Formate	0.01812	0.00984	0.0201	0.0129	0.00658	0.01456	0.0147	0.01596	0.04	0.0151	0.01156	0.04032
Lactate	2.69954	2.5096	2.1128	2.51718	2.5228	1.1212	2.02842	0.80668	1.40506	2.35278	0.78222	0.49882
Asparagine	0	0.02706	0.02422	0	0	0	0	0.04136	0	0	0.00434	0
Glycine	0.07006	0.08476	0.02988	0.00906	0.04068	0.01	0.02506	0.01744	0.02866	0.02914	0.0116	0.01154
Leucine	0.05828	0.01576	0.06156	0	0.01022	0.01318	0.03182	0.0228	0.03724	0.037	0.02558	0.01458
Tyrosine	0	0.00416	0.0238	0	0	0.00204	0.00442	0.00508	0	0	0	0
Isoleucine	0.01492	0.003	0.01842	0	0	0.00406	0.014	0.00584	0.01362	0.02268	0.00796	0.00536
Aspartate	0.0183	0.02082	0.00814	0	0.01056	0.00622	0.00956	0	0.0096	0.01198	0.00676	0
Glutamate	0	0	0.02888	0	0.03238	0	0.0249	0	0	0.03136	0	0
Glutamine	0	0	0.01102	0	0	0	0	0	0	0	0	0
Alanine	0.08846	0.04926	0.0801	0.0387	0.03644	0.02232	0.08132	0.03632	0.05096	0.10138	0.03168	0.03466
Threonine	0.02406	0.01636	0.0195	0	0	0.01092	0	0.017	0.0132	0.00538	0.00464	0
4-Aminobutyrate	0.07246	0.0479	0.05966	0.02044	0.04892	0.01186	0.03348	0.03942	0.0372	0.03972	0.02168	0.0238
Valine	0.03738	0.015	0.03896	0.00172	0.0128	0.01084	0.02938	0.01384	0.02428	0.0349	0.01578	0.01168
Methionine	0.0132	0.00406	0.015	0	0.00288	0.00252	0.00626	0.00474	0.0077	0.00926	0.0046	0.00322
Tryptophan	0.00508	0.00158	0.0054	0.00166	0.00264	0	0.00254	0.00194	0.00176	0.00462	0	0
Phenylalanine	0.02506	0.00572	0.02092	0	0	0.00624	0.01278	0.01124	0.0164	0.01632	0.01122	0.00434
Fructose	0	0	0	0	0	0	0	0.03716	0.07664	0	0	0.01552
Sucrose	0	0	0	0	0	0	0.04834	0	0.04292	0.04528	0	0.16696
Galactose	0.09584	0.11886	0.06338	0	0	0	0	0.02268	0	0	0	0
Arabinose	0	0	0	0	0	0	0	0	0	0	0	0
Mannose	0	0	0	0	0	0	0	0	0	0	0	0
Glucose	0	0.14196	0.03872	0	0.0186	0.01666	0.00894	0.64606	0.09102	0.0071	0	0.0074
Maltose	0	0	0	0	0.13184	0	0	0.01818	0	0.01718	0	0
Betaine	0.003708	0.0012	0.002864	0.00046	0.0036	0.011684	0.0017	0.001692	0.004776	0.0012	0.002056	0.00116
Trigonelline	0.001152	0.000284	0.000796	0.000256	0.00088	0.001176	0.000496	0.000408	0.000632	0	0.000632	0.000296
Uracil	0.002736	0.00082	0.002008	0.000696	0.001872	0.002144	0	0.001436	0.002384	0	0.0018	0.0004
Choline	0.014116	0.00306	0.010748	0.002388	0.01104	0.010892	0.007244	0.006848	0.008404	0.005344	0.007904	0.004656
Succinate	0.024296	0.00012	0.02098	0.00022	0.013864	0.032132	0.0142	0.0053	0.004732	0.009864	0.004948	0.006932

Using two-way ANOVA with Tukey's multiple comparisons test showed that the concentrations of metabolites produced showed significant differences (p-value=0.0002) whilst some others did not show any significant differences between the different processing stages as shown in Table 2 (a to d). * means significant at p = 0.05 (significant), **** means significant at p = 0.0001 (highly significant) were used as the cut-off values. For instance, for organic compounds, there were significant changes in the concentration of metabolites depending on the production stages. Generally, glucose concentration was significantly different between all paired group fermentation stages. Acetate concentration was significantly different between grouped D vs 12 h (p-value <0.0001), D vs 24 h (p-value = 0.0346) and D vs M (P-value < 0.0001). Similarly, at these same production stages, as well as D vs Su, 12 h vs Su, 12 h vs K, 24 h vs. Su, 24 h vs. K and M vs. K, lactate concentrations in total were significantly different (p-value < 0.0001; = 0.0354; <0.0001; 0.0046; <0.0001 and <0.0001 respectively). Similar trends were observed with the concentrations of amino acids, sugars and other key metabolites as shown in Table 2 (a to d). Figures 10a to 10c also shows organic acid trends observed at three processing stages (D, M and K).

Table 2: Concentration (mmol/100 g) of selected metabolites compared between two fermentation stages

2a

Sugars/Paired Stages	Mean 1	Mean 2	Mean difference	Significant difference	P. Value
Glucose					
D vs. 12 h	6.898	3.205	3.692	****	<0.0001
D vs. 24 h	6.898	4.234	2.664	**	0.0023
D vs. M	6.898	7.23	-0.3321	ns	0.9791
D vs. Su	6.898	0.09464	6.803	****	<0.0001
D vs. K	6.898	0.1428	6.755	****	<0.0001
12 h vs. 24 h	3.205	4.234	-1.029	ns	0.7121
12 h vs. M	3.205	7.23	-4.024	****	<0.0001
12 h vs. Su	3.205	0.09464	3.111	****	<0.0001
12 h vs. K	3.205	0.1428	3.063	****	<0.0001
24 h vs. M	4.234	7.23	-2.996	***	0.0003
24 h vs. Su	4.234	0.09464	4.139	****	<0.0001
24 h vs. K	4.234	0.1428	4.091	****	<0.0001
M vs. Su	7.23	0.09464	7.135	****	<0.0001
M vs. K	7.23	0.1428	7.087	****	<0.0001
Su vs. K	0.09464	0.1428	-0.04817	ns	>0.9999
Fructose					
D vs. 12 h	3.48	0.1393	3.341	****	<0.0001
D vs. 24 h	3.48	0.08438	3.396	****	<0.0001
D vs. M	3.48	0.6303	2.85	****	<0.0001
D vs. Su	3.48	0.003749	3.476	****	<0.0001
D vs. K	3.48	0.01726	3.463	****	<0.0001
12 h vs. 24 h	0.1393	0.08438	0.05489	ns	>0.9999
12 h vs. M	0.1393	0.6303	-0.4911	ns	0.9177
12 h vs. Su	0.1393	0.003749	0.1355	ns	0.9998
12 h vs. K	0.1393	0.01726	0.122	ns	0.9999
24 h vs. M	0.08438	0.6303	-0.546	ns	0.9701
24 h vs. Su	0.08438	0.003749	0.08063	ns	>0.9999
24 h vs. K	0.08438	0.01726	0.06711	ns	>0.9999
M vs. Su	0.6303	0.003749	0.6266	ns	0.7345
M vs. K	0.6303	0.01726	0.6131	ns	0.7523
Su vs. K	0.003749	0.01726	-0.01351	ns	>0.9999

2b

Organic acids/Paired Stages	Mean 1	Mean 2	Mean difference	Significant difference	P. Value
Lactate					
D vs. 12 h	0.1201	6.064	-5.944	****	<0.0001
D vs. 24 h	0.1201	7.793	-7.673	****	<0.0001
D vs. M	0.1201	5.723	-5.603	****	<0.0001
D vs. Su	0.1201	3.648	-3.528	****	<0.0001
D vs. K	0.1201	1.701	-1.581	ns	0.2963
12 h vs. 24 h	6.064	7.793	-1.729	ns	0.6947
12 h vs. M	6.064	5.723	0.3411	ns	0.9983
12 h vs. Su	6.064	3.648	2.416	*	0.0354
12 h vs. K	6.064	1.701	4.363	****	<0.0001
24 h vs. M	7.793	5.723	2.071	ns	0.4641
24 h vs. Su	7.793	3.648	4.145	**	0.0046
24 h vs. K	7.793	1.701	6.092	****	<0.0001
M vs. Su	5.723	3.648	2.075	ns	0.0617
M vs. K	5.723	1.701	4.021	****	<0.0001
Su vs. K	3.648	1.701	1.947	ns	0.0962
Acetate					
D vs. 12 h	0.167	4.035	-3.868	****	<0.0001
D vs. 24 h	0.167	3.627	-3.46	*	0.0346
D vs. M	0.167	5.819	-5.652	****	<0.0001
D vs. Su	0.167	0.7608	-0.5938	ns	0.9702
D vs. K	0.167	0.2827	-0.1156	ns	>0.9999
12 h vs. 24 h	4.035	3.627	0.4081	ns	0.9994
12 h vs. M	4.035	5.819	-1.784	ns	0.2382
12 h vs. Su	4.035	0.7608	3.274	***	0.0009
12 h vs. K	4.035	0.2827	3.752	****	<0.0001
24 h vs. M	3.627	5.819	-2.192	ns	0.3974
24 h vs. Su	3.627	0.7608	2.866	ns	0.1272
24 h vs. K	3.627	0.2827	3.344	*	0.0434
M vs. Su	5.819	0.7608	5.058	****	<0.0001
M vs. K	5.819	0.2827	5.536	****	<0.0001
Su vs. K	0.7608	0.2827	0.4781	ns	0.9877

2c

Amino acids/Paired Stages	Mean 1	Mean 2	Mean difference	Significant difference	P. Value
Leucine					
D vs. 12 h	0.108	0.7381	-0.6302	****	<0.0001
D vs. 24 h	0.108	1.017	-0.9094	****	<0.0001
D vs. M	0.108	0.3741	-0.2662	****	<0.0001
D vs. Su	0.108	0.05731	0.05067	ns	0.9162
D vs. K	0.108	0.02685	0.08113	ns	0.5923
12 h vs. 24 h	0.7381	1.017	-0.2792	**	0.0059
12 h vs. M	0.7381	0.3741	0.364	****	<0.0001
12 h vs. Su	0.7381	0.05731	0.6808	****	<0.0001
12 h vs. K	0.7381	0.02685	0.7113	****	<0.0001
24 h vs. M	1.017	0.3741	0.6432	****	<0.0001
24 h vs. Su	1.017	0.05731	0.96	****	<0.0001
24 h vs. K	1.017	0.02685	0.9905	****	<0.0001
M vs. Su	0.3741	0.05731	0.3168	****	<0.0001
M vs. K	0.3741	0.02685	0.3473	****	<0.0001
Su vs. K	0.05731	0.02685	0.03046	ns	0.99
Alanine					
D vs. 12 h	0.2178	1.243	-1.026	****	<0.0001
D vs. 24 h	0.2178	1.383	-1.165	****	<0.0001
D vs. M	0.2178	0.8093	-0.5916	****	<0.0001
D vs. Su	0.2178	0.1152	0.1026	ns	0.3227
D vs. K	0.2178	0.0532	0.1646	*	0.0143
12 h vs. 24 h	1.243	1.383	-0.1398	ns	0.4889
12 h vs. M	1.243	0.8093	0.434	****	<0.0001
12 h vs. Su	1.243	0.1152	1.128	****	<0.0001
12 h vs. K	1.243	0.0532	1.19	****	<0.0001
24 h vs. M	1.383	0.8093	0.5738	****	<0.0001
24 h vs. Su	1.383	0.1152	1.268	****	<0.0001
24 h vs. K	1.383	0.0532	1.33	****	<0.0001
M vs. Su	0.8093	0.1152	0.6942	****	<0.0001
M vs. K	0.8093	0.0532	0.7561	****	<0.0001
Su vs. K	0.1152	0.0532	0.06197	ns	0.8112

2d

Other key metabolites/Paired Stages	Mean 1	Mean 2	Mean difference	Significant difference	P. Value
Choline					
D vs. 12 h	0.3192	0.2217	0.09743	****	<0.0001
D vs. 24 h	0.3192	0.2626	0.05658	ns	0.1316
D vs. M	0.3192	0.3139	0.005243	ns	0.9993
D vs. Su	0.3192	0.04058	0.2786	****	<0.0001
D vs. K	0.3192	0.008117	0.311	****	<0.0001
12 h vs. 24 h	0.2217	0.2626	-0.04086	ns	0.5048
12 h vs. M	0.2217	0.3139	-0.09219	****	<0.0001
12 h vs. Su	0.2217	0.04058	0.1811	****	<0.0001
12 h vs. K	0.2217	0.008117	0.2136	****	<0.0001
24 h vs. M	0.2626	0.3139	-0.05133	ns	0.2088
24 h vs. Su	0.2626	0.04058	0.222	****	<0.0001
24 h vs. K	0.2626	0.008117	0.2545	****	<0.0001
M vs. Su	0.3139	0.04058	0.2733	****	<0.0001
M vs. K	0.3139	0.008117	0.3058	****	<0.0001
Su vs. K	0.04058	0.008117	0.03247	ns	0.234
Betaine					
D vs. 12 h	0.2171	0.1028	0.1143	****	<0.0001
D vs. 24 h	0.2171	0.09013	0.127	****	<0.0001
D vs. M	0.2171	0.1096	0.1075	****	<0.0001
D vs. Su	0.2171	0.01607	0.201	****	<0.0001
D vs. K	0.2171	0.003215	0.2139	****	<0.0001
12 h vs. 24 h	0.1028	0.09013	0.01266	ns	0.9945
12 h vs. M	0.1028	0.1096	-0.00683	ns	0.9982
12 h vs. Su	0.1028	0.01607	0.08671	****	<0.0001
12 h vs. K	0.1028	0.003215	0.09957	****	<0.0001
24 h vs. M	0.09013	0.1096	-0.01948	ns	0.9552
24 h vs. Su	0.09013	0.01607	0.07405	*	0.0146
24 h vs. K	0.09013	0.003215	0.08691	**	0.002
M vs. Su	0.1096	0.01607	0.09353	****	<0.0001
M vs. K	0.1096	0.003215	0.1064	****	<0.0001
Su vs. K	0.01607	0.003215	0.01286	ns	0.9519

NB: ns = not significant

*/**/***/**** = Significant difference

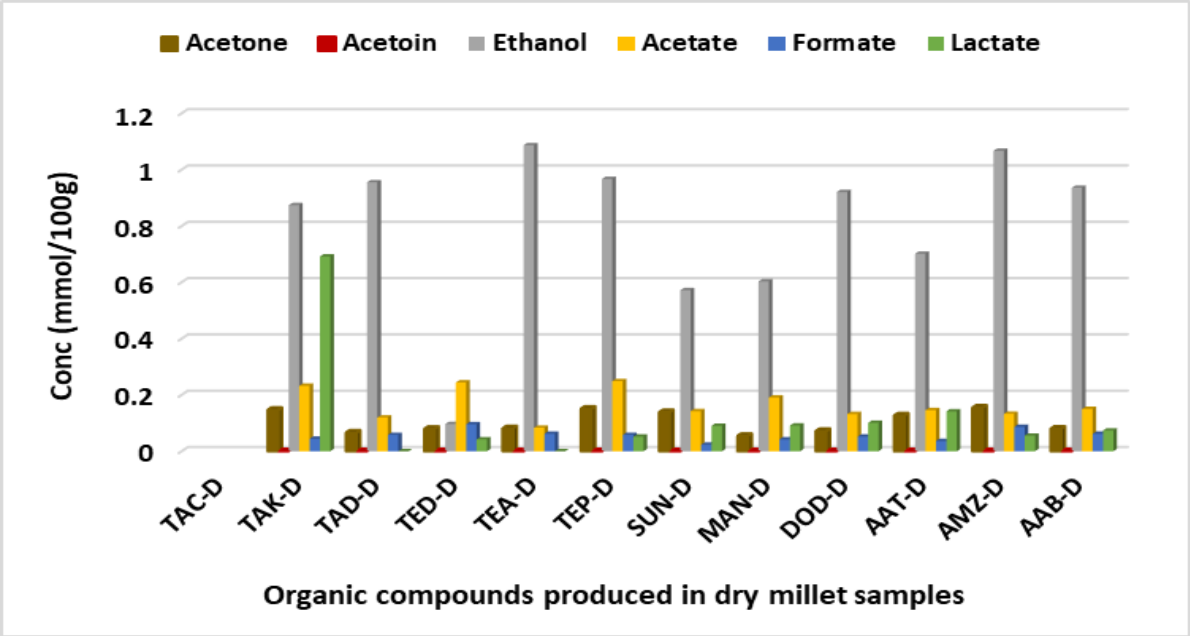


Figure 10a: Organic compounds produced from dry millet grains for *Hausa koko* production from commercial processors

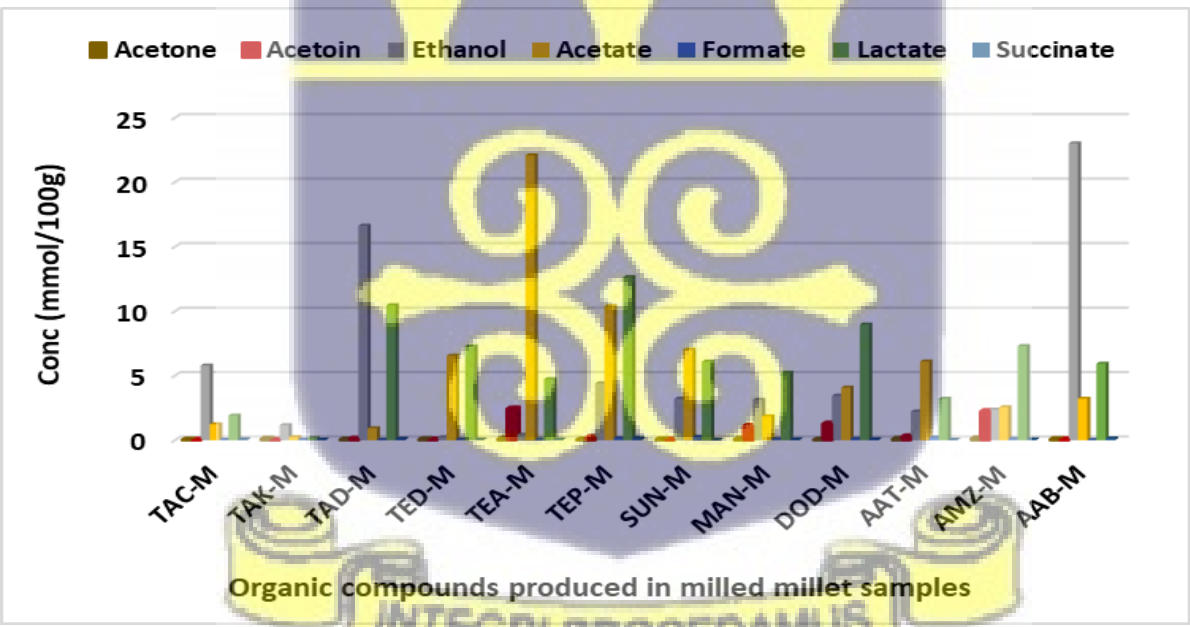


Figure 10b: Organic compounds produced from milled millet with spices for *Hausa koko* production from commercial processors

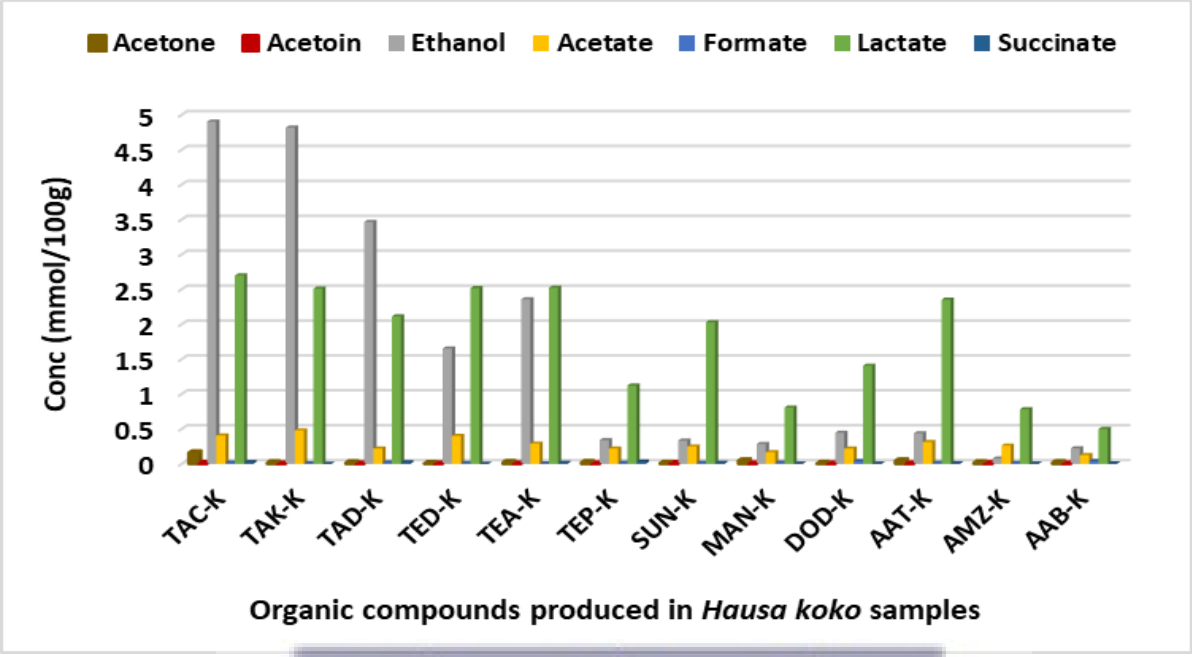


Figure 10c: Organic compounds produced from *Hausa koko* from commercial processors

NB:

Sampling points:

D = dry millet grains; M = milled millet with spices; K = *Hausa koko*

Sampling sites:

Tamale Central (TAC); Tamale Kalariga (TAK); Tamale Dabokpa (TAD); Techiman Diasempa (TED); Techiman Abourso (TEA); Techiman Pomaakrom (TEP); Sunyani (SUN); Mankessim (MAN); Dodowa (DOD); Accra Ashaiman-Tulaku (AAT); Accra Madina Zongo (AMZ); Accra Asharley Botwe (AAB).

3.4 Discussion

3.4.1 Bacterial diversity

Different culture-independent methods have been used in identifying the microbial diversity of many fermented foods globally including millet (Qin et al., 2016), maize (Assohoun-djeni et al., 2016; Wakil et al., 2008), milk (Shangpliang et al., 2018), soy sauce (Yan et al., 2013), sausages (Cocolin et al., 2011). In this study, the bacterial diversity was also successfully explored with high throughput Illumina HiSeq sequencing of the V4 hypervariable region of the 16S rRNA gene amplicons. The species richness obtained from the different stages of *Hausa koko* production was a clear indication of the abundance and variety of bacteria that existed at the different processing stages. The milled millet with spices (M), supernatant (Su), sediments (Sd) and *Hausa koko* (K) samples shared a total of 135 operational taxonomic units (OTUs) or closely related species together. Thus 135 different bacteria were common among these fermenting stages.

Irrespective of the geographical location they were obtained from, the samples were generally similar at the same processing stages in terms of the bacterial diversity, which may be attributed to the processors following the same production process and the use of the same or similar raw materials. The alkaline nature of the millet grains might have accounted for the high abundance of bacteria that were observed in them with a relatively higher population of the bacteria genus *Pantoea*. Most of the Gram-negative bacteria including *Pseudomonas, Chryseobacterium, Bacteroides, Sphingomonas, Escherichia-Shigella, Enterbacteriaceae, Staphylococcus, Serratia,* etc dominating the grain samples are known potential pathogens (Azizi et al., 2020; Gadaga et al., 2004). Some of these organisms may be inherently associated with the raw materials in their natural environment. These may be an indication of faecal, soil and environmental contamination.

Some strains of these organisms may cause spoilage whilst others have been implicated with infections and diseases such as nausea, vomiting, gastroenteritis, cholera, typhoid fever and diarrhoea raising public health concerns (Gadaga et al., 2008; 2004). Contamination may be attributed to poor handling, inadequate safety measures, poor manufacturing practices, poor storage system, and poor sanitary conditions. Others including contaminants in the air, under the nails of attendants, on cloths, body, cooking utensils, water sources, have been reported (Oyelana & Coker, 2012; Yagoub, 2009; Gadaga et al., 2008; 2004; Hardalo & Edberg, 1997).

There was however a major shift in the bacterial community from the grains to 12 h fermented millet especially, and the rest of the fermentation processing stages (24 h, M, Su and Sd). The abundance of all the fermentation related genera increased significantly whilst the potential pathogenic, plant and environmental related genera reduced. The production of organic acids by the fermenting microorganisms lowered the pH from 5.45-6.58 to 3.51-3.99 which caused a reduction in the population of the Gram-negative bacteria group (Owusu-Kwarteng et al., 2012). Synthesis of metabolites such as organic acids by lactic acid bacteria (LAB) and acetic acid bacteria (AAB) groups during the key fermentation stages may have also accounted for the significant differences observed (Achi & Ukwuru, 2015; Owusu-Kwarteng et al., 2012; Lei & Jakobsen, 2004). The fermentation stages were dominated by LAB groups mostly *Lactobacillus* (now *Limosilactobacillus*). Others including *Pediococcus, Weissella, Lactococcus, Streptococcus, Acetobacter* and *Leuconostoc* also dominated. Their abundance however was reduced in the *Hausa koko* samples across the six regions which might be due to higher volumes of dilution with water and heat application during cooking. Different culture dependent and independent studies of cereal fermented products have also reported similar trends of these LAB genera in cereals such as millet,

maize, and sorghum (Henshaw et al., 2016; Assohoun-djeni et al., 2016; Annan et al., 2015; Okeke et al., 2015; Owusu-kwarteng et al., 2012; Oguntoyinbo & Narbad, 2012; Oguntoyinbo et al., 2011; Kalui et al., 2009; Vieira-Dalode´et al., 2007; Lei & Jakobsen, 2004; Halm et al., 1993). This result is also similar to those reported between all processing stages for different formulations of *kunu*, a traditional cereal based fermented beverage (Ezekiel et al., 2019). Diaz et al., (2019) also confirmed the dominance of the genera within the order *Lactobacillales* in fermented cereal, dairy, and cassava products from eight different African countries using 16S rRNA gene amplicon sequencing.

Acetobacter and *Gluconobacter* were present in different levels of relative abundance at all the stages of *Hausa koko* production including the final product where they had the highest abundance. Both are Gram negative Acetic Acid Bacteria (AAB), known for the production of acetic acid, vitamin C, cellulose and associated with spontaneous and backslopped fermentation of foods and beverages such as cocoa, kefir, kombucha and other beers (De Roos & De Vuyst, 2018). Their strict aerobic nature and resistance to low pH and ethanol may, to some extent, have promoted their ability to persist and thrive during *Hausa koko* production (Gullo et al., 2014). This result is similar to others reported in other cereal fermentations like *burukutu* (Oguntoyinbo, 2014) and *kunu* (Ezekiel et al., 2019) where *Gluconobacter* was reportedly responsible for the oxidation of the ethanol produced during the fermentation to acetic acid (Gómez-Manzo et al., 2010).

Significant differences that existed between different processing stages was not surprising as each stage was unique and introduced its own microbial community. The bacterial diversity of samples within the same processing stage as well as among processors of the same Region were largely the

same. However, the few differences that existed from different processors as observed for instance with *Hausa koko* samples from Accra and Techiman and also between Tamale and Techiman may be attributed to several reasons. (i) These differences may have arisen from the microbes associated with the dry millet as these millets are cultivated from different parts of the Northern Regions on soils with different microbial communities, handling practices and probably differences in varieties as found in various markets. (ii) The mode of processing the millet is sometimes unhygienic as it may be done on the bare floor contaminating the grains with microbes from sand, stones and other foreign materials, therefore potentially accounting for the significant difference between it and other time points or stages. (iii) Another factor may be the quality of water used for cooking as the *Hausa koko* samples contain considerably more water than all the other stages of processing, however the bacterial diversity in the water used was not analysed in this study. (iv) The microbial communities of spices, utensils and other contact surfaces used could have also influenced the outcomes (Gadaga et al., 2008).

The differences found between samples from the Northern Region and the rest of the other Regions were attributed to the presence of rod-shaped lactic acid producing *L. helveticus.* They have been reported in dairy fermentation and intestinal microflora as having potential probiotic properties (Chen et al., 2014; Zhao et al., 2011; Frece et al., 2009). Its pure culture has also been used in the fermentation of soy beverage and fermented foods like *kimchi* and *kombucha* but not widely reported with cereal fermentation (Felix, 2016; Champagne et al., 2010). Its presence in the Northern Region samples may be due to contamination with cow milk which is abundant in that part of Ghana and also due to the high temperatures existing in this area which might have promoted their proliferation as they are thermophilic in nature (Gatti et al., 2004). This assertion

is confirmed by Akabanda et al., (2014) who reported its presence, probiotic potential and usage for starter culture development in *nunu*, a spontaneously fermented cow milk product from Northern Ghana.

3.4.2 Metabolites produced

Metabolites profiling performed by nuclear magnetic resonance spectroscopy (NMR) was able to identify metabolites formed at the different stages of *Hausa koko* production. Samples analysed from twelve (12) different commercial processors located in six (6) different regions of Ghana showed similar metabolite production in varying concentrations. The metabolites were produced from the microbial communities existing at each specific stage of production and their interactions. The similarities observed in the types of metabolites profiled within and between regions may be as a result of these processors using similar raw materials. The differences in few of the types occurring and varying concentrations however may arise from the composition of individual raw materials mainly millet, spices, water, cooking utensils and the diversity and concentrations of microbes they carried (Akpinar-Bayizit et al., 2010; Jespersen, 2003). The quantity of spices for instance used by a traditional processer is not measured or standardized. They normally fetch with the hand and add based on experience. This practice will definitely influence the concentration of metabolites from one processor to the other.

A general reduction in sugars and increase in organic compounds was observed. Fermentative organic compounds such as lactate, acetate and ethanol production progressed steadily along the fermentation stages peaking in the milled millet samples and further in the supernatants. Their concentrations subsequently reduced in all the final *Hausa koko* samples analyzed due to dilution

with water during preparation. These organic compounds were produced by the dominating LAB groups mostly *Lactobacillus* (now *Limosilactobacillus*) and others like *Pediococcus, Weissella, Lactococcus, Streptococcus* and *Leuconostoc* from the fermentation stages. For cereal fermentation like this, yeast are expected to be the other dominating microbes even though yeast community was not reported here (Ogunremi et al., 2015; Greppi et al., 2013; Owusu-Kwarteng et al., 2010; Michodjèhoun-Mestres et al., 2005). The profiling of more metabolites from the fermenting stages, milled and supernatant samples may be a result of increase in the population of these LAB. Some of the bacteria identified may be heterofermenters and may have synthesized lactic acid in addition to other organic compounds identified. They transformed the available sugars into organic compounds such as lactate, acetate, ethanol, formate, succinate, acetone and acetoin. The presence of the different spices may have also contributed to the formation of some of these metabolites (Moratalla-López et al., 2019). The high concentrations of ethanol in some of the *Hausa koko* samples, especially those from the Northern Region, may be attributed to excess fermentation. According to Akpinar-Bayizit et al., (2010), the composition of organic compounds is affected by the fermentation process as well as the raw material composition of the food. Formation of such organic compounds have been reported in other fermented foods. Lactate, acetate formate, succinate were formed during fermentation by *L. pentosus* (Cselovszky et al., 1992). *L. helveticus* ATCC15807 was also reported to have produced acetate and succinate during fermentation (Torino et al., 2005). Under anaerobic conditions, *Escherichia coli* is also reported to produce succinate as a minor fermentation compound (Lin et al., 2005). Akpinar-Bayizit et al., (2010) reported the production of alcohol, lactic, acetic, oxalic and citric acids in *Boza* and suggested these organic compounds contributed to the sensory properties of the beverage. Other

reports have confirmed the flavor development properties of organic compounds in cereal foods (Weldemichael et al., 2019; Onyango et al., 2000; Sripriya et al., 1997; Cselovszky et al., 1992).

Despite some erratic trends in the concentration of sugars along the production stages, there was a general reduction in their concentration. Cereals are composed mainly of starch, water soluble and insoluble components of fiber in addition to different sugars. This makes them suitable raw materials for fermentation as well as a good source of carbon for the microorganisms (Di Stefano et al., 2017; Charalampopulos et al., 2002). Glucose, the most dominant among the sugars was more pronounced in the milled samples which may be attributed to the milling process. This potentially reduced the particle size of the grains making them readily available for enzymes to breakdown into simple sugars for the fermenting microbes to utilize. The microbial activity by the fermenting microbes consequently converted the substrate starch and sugars into organic compounds mainly lactate, acetate ethanol and other flavour compounds accounting for their reduction (Furukawa et al., 2013). Dilution with water resulted in the reduction of the sugar concentrations in the supernatant and *Hausa koko*.

Amino acids and their intermediary compounds were another group of important metabolites profiled. Their occurrence was anticipated since cereals like millet are known to contain some nominal amount of amino acids (Amadou et al., 2013). This included leucine, isoleucine, tryptophan, valine, methionine, alanine and many more. It was observed that their concentrations generally varied along the processing stages among all processors with some either reducing or not detected at all. Their concentrations in the grain samples were largely low but improved marginally in the 12 h fermented millet and some milled samples, suggesting some level of

increment between these three stages (Adebiyi et al., 2017; Mbithi-Mwikya et al., 2000). Amino acid increases have been ascribed to an elevated population of hydrolytic enzymes which hydrolyses the protein (Saleh et al., 2013). Obviously due to dilution with water in the supernatant and *Hausa koko* samples, their concentrations reduced. Other important metabolites such as choline, uracil, trigonelline and betaine in very low concentrations were also identified. They play key roles such as amino acid metabolism, lipid metabolism, decreases in blood cholesterol levels and many others (Bahmani et al., 2016; Ross et al., 2014; Bruce et al., 2010; Basch et al., 2003).

Fermentative metabolites have been reported in cereal foods. Most of these studies used different methods including high performance liquid chromatography (HPLC), liquid chromatography-tandem mass spectrometry and automatic static headspace gas chromatography (HS GC). Twenty one (21) different metabolites were profiled during the fermentation of a Tanzanian fermented cereal food, *togwa*. Some of these included lactates, succinate, pyruvate, formate, citrate, with other volatile organic compounds such as 2-methyl-1-butanol, acetoin, and many others. Ethanol, the predominant compound produced increased with fermentation time. The sugars, maltose, fructose and glucose were also identified in *togwa* (Mugula et al., 2003a). Kojic acid, cyclopiazonic acid, nidurufin, helvolic acid and several other fungal metabolites were identified in *oshikundu*, a popular Namibian sorghum and pearl millet beverage (Misihairabgwi et al., 2018). In *obushera*, another fermented millet and/or sorghum beverage, the presence of twenty two (22) metabolites were reported. These were categorized under organic acids, sugars, alcohols and volatile compounds where these were attributed to contribute to flavor development (Mukisa et al., 2012). It is therefore suggested that similarly, the metabolites produced through microbial

interactions and activities present during *Hausa koko* production may also help in the development of its flavor and aroma (Weldemichael et al., 2019; Salmerón et al., 2014).

3.5 Conclusion

The bacterial diversity during *Hausa koko* production has been established and this study provides the most comprehensive bacterial profile (over 400) yet reported at all processing stages of *Hausa koko*. Significant differences existed between samples at different processing stages. Samples within the same processing stage were mostly not significantly different. The same was observed among processors of the same region. A wide variety of Gram positive and negative bacteria of the genus *Pantoea* dominated the grain samples. The Gram negatives were dominated by potential pathogenic bacteria including Enterobacteriaceae, *Staphylococcus, Escherichia-Shigella* among others, but their population reduced progressively along the fermentation stages which were dominated by lactic acid bacteria (LAB). The LAB included species of *Lactobacillus* (now *Limosilactobacillus*), *Pediococcus, Weissella, Lactococcus, Streptococcus* and *Leuconostoc.* Acetic acid producing *Acinobacter* and *Gluconobacter* were present at all processing stages. Samples from the Northern Region were significantly different from the samples from other Regions due to OTUs classified as *L. helveticus*. The metabolomics study using NMR unveiled a comprehensive profile of measurable metabolites occurring during *Hausa koko* fermentation processes. A total of 33 different metabolites were identified and classified as organic compounds including alcohols, sugars, amino acids and some other key compounds. The different fermenting microorganisms mainly lactic acid bacteria identified at the fermentation stages produced the various metabolites. These metabolites may also influence the flavor and other unique sensorial attributes of *Hausa koko*.

CHAPTER FOUR

4.0 Lactic acid bacteria and yeasts associated with the traditional fermentation of millet in *Hausa koko* production

4.1 Introduction

Maize, millet, sorghum, rice, wheat, and many other cereals used in fermentation are very good substrates for many microorganisms (Tamang et al., 2016; Corsetti & Settanni, 2007). Their fermentation is mostly started by mixed population of microbes originating from the raw materials, process equipment, fermenting receptacle, the environment, backslopping product and many more (Jespersen et al., 1994). The microbial ecology is thus composed of diverse interrelated microorganisms associated with the raw material and production process, but only few of them essentially determine the quality of the final product (Abegaz, 2007). This is because, as their fermentation progresses, the increasing acidity from fermenting microorganisms, mainly lactic acid bacteria (LAB) eliminates non-lactic acid microbes. They also inhibit some neutrophiles including some pathogenic and spoilage organisms, whilst the remaining surviving LAB form synergy with yeast species. Microbial succession could also be a reason, whereby the ecology of the microbes that begin the fermentation process will differ from those ending the fermentation, especially for prolonged fermentation (Achi & Ukwuru, 2015).

LAB are Gram positive, catalase negative, facultative anaerobes, non-respiring, non-sporing rods or cocci which produce mainly lactic acid and other organic compounds as the end product during carbohydrate fermentation (Mani, 2018; Makarova et al., 2006; Axelsson, 2004). They are mostly mesophilics that can grow below 4 °C and above 45 °C, in a pH range of 4.0 – 4.5. Others can also

grow either in high pH above 9.0 or as low as 3.2. Their optimum pH is however around 5.5 – 6.5 and 30 °C for growth temperature (Bamforth, 2005; Mayra-Makinen & Bigret, 2004).

Yeast species are among the most valuable microorganisms used for biotechnological purposes for economic revenue, not just for food fermentation purposes but also for the production of fuel, ethanol, small molecular weight metabolites, feed and fodder (Johnson, 2013). The most well-known yeast species is *Saccharomyces cerevisiae* which happens to be the most common isolated yeast associated with fermented foods and beverages (Bourdichon et al., 2012; Jespersen, 2003). Other *Saccharomyces* species used in biotechnological processes are *S. cariocanus*, *S. kudriavzevii*, *S. paradoxus*, *S. mikatae* and *S. aboricolus*. In addition, non-Saccharomyces yeast genera including *Candida, Pichia, Kluyveromyces, Torulaspora* among others are all found in the environment and associated with food fermentations playing key role during the fermentation process (Roudil et al., 2020; Vaughan-Martini & Martini, 2011).

The presence of various LAB and yeast species have been reported in African fermented foods (Houngbédji et al., 2018; Greppi et al., 2017; Assohoun-Djeni et al., 2016; Oguntoyinbo & Narbad 2015, 2012; Annan et al., 2015; Atter et al., 2014; Akabanda et al., 2014; Nyanga et al., 2007; Omemu et al., 2007; Abriouel et al., 2006; Jespersen et al., 2005; Amoa-Awua et al., 1997). These studies used phenotypic and genotypic methods for identification of microorganisms. The genotypic methods used involved mostly sequencing of specific hyper variable regions. There is little information on the use of whole genome sequencing (WGS) in the study of indigenous African fermented foods. However, such an approach to the study of microbial isolats from traditional fermentation processes will not only identify fermenting organisms but also project their functionality leading to selection of beneficial specific traits for commercial exploitation including their use in starter culture development.

In studies of spontaneously fermented sour products in Africa, yeasts have mostly been reported to play a key role in the fermentation alongside the lactic acid bacteria, which is responsible for the souring of the product. The yeasts are reported to contribute to the flavour of the product and facilitate growth of the LAB. Yeasts involved in the fermentation of *Hausa koko* have not been reported although that of LAB using 16S rRNA gene sequencing approach has been reported (Lei & Jakobsen, 2004). A combination of phenotypic and current high throughput Next Generation Sequencing methods that have high discriminatory power, accuracy and sensitivity can be explored in this regard. Additionally, the use of bioinformatics tools will help provide comprehensive information about these fermenting microorganisms. This work was carried out as an in-depth study of the lactic acid bacteria and yeast species involved in the fermentation of millet during *Hausa koko* production from five regions of Ghana using whole genome sequencing (WGS) for lactic acid bacteria and Sanger sequencing for yeast respectively.

4.2 Materials and Methods

4.2.1 Sampling

The study was conducted using sample products at different stages of production collected from five processing sites located in five political regions of Ghana. These were from the Tamale Dabokpa (TAD) in the Northern Region; Sunyani (SUN) in the Bono Region: Mankessim (MAN) in the Central Region; Dodowa (DOD) in the Eastern Region, and Accra Madina Zongo (AMZ) in the Greater Accra Region. The samples collected from each production site were dry millet grains (D), 12 h steeping millet (12 h), 24 h steeping millet (24 h), milled millet with spices (M), fermented slurry - supernatant (Su), fermented slurry- sediment (Sd) and *Hausa koko* (K). Samples were collected aseptically into sterile sampling containers and transported to CSIR-Food Research Institute (FRI) in Accra under cold storage where they were preserved at -20 °C. They were transported under cold storage to Quadram Institute Bioscience (QIB), Norwich, UK.

4.2.2 Determination of pH

The pH of samples were determined as previously described (Chapter 3; 3.2.3.1).

4.2.3 Microbiological analysis

4.2.3.1 Preparation of serial dilutions

For enumeration and isolation of lactic acid bacteria, lactococcus and yeasts, one gram of sample was added to 9 ml of sterile PBS solution with pH adjusted to 7.2 and vortexed for 30 s at normal speed. From appropriate ten-fold dilutions, 100 µl aliquots of each dilution was inoculated onto

the appropriate solid media for enumeration and isolation of *Lactobacillus*, *Lactococcus* and yeasts (Diaz et al., 2020). For enumeration of aerobic mesophiles, coliforms, *E. coli*, *Bacillus cereus*, *Staphylococcus aureus* and Enterobacteriaceae, 10 g of sample was added to 90 ml of Salt Peptone Solution (SPS) containing 0.1 % peptone and 0.85 % NaCl with pH adjusted to 7.2, and serial dilutions prepared with 1 ml aliquots following standard methods by Nordic Committee on Food Analysis (NMKL).

4.2.3.2 Enumeration of aerobic mesophilic bacteria

The population of aerobic mesophiles was enumerate on Plate Count Agar (Oxoid CM325, Oxoid Ltd., Basingstoke, Hampshire, UK) using the pour plate method (NMKL No. 86, 1999). Plates were incubated at 30 °C for 72 h.

4.2.3.3 Enumeration of LAB

The spread plate method was used in the enumeration of *Lactobacillus* using deMan, Rogosa and Sharpe (MRS, Oxoid CM359, Oxoid Ltd., Basingstoke, Hampshire, UK.) with 10 % agar (AGA03, Formedium Ltd, UK) added according to De Man et al., (1960), pH 6.2. The media was supplemented with 0.1 % cycloheximide (A0406195, China) to inhibit the growth of yeast and incubated aerobically at 37 °C for 2-3 d. For the enumeration of *Lactococcus* species M17 (Oxoid CM 0817, Oxoid Ltd., Basingstoke, Hampshire, UK.) with agar (AGA03, Formedium Ltd, UK) and sterile 10 % lactose solution was used (Diaz et al., 2020).

4.2.3.4 Enumeration of yeast

Enumeration of yeast was done by the spread plate method using Rose Bengal Chloramphenicol Agar (Oxoid CM 0549 Oxoid Ltd., Basingstoke, Hampshire, UK) pH 5.5 to which chloramphenicol (C0113.0025, Duchefa Biochemie, Netherlands) was added to inhibit the growth of bacteria. The plates were incubated at 25 °C for 3-5 d.

4.2.3.5 Enumeration of enterobacteriaceae

Enterobacteriaceae was enumerated on Tryptone Soya Agar (Oxoid CM 131, Oxoid Ltd., Basingstoke, Hampshire, UK), pH 7.3, overlaid with Violet Red Bile Glucose Agar (Oxoid CM 0485, Oxoid Ltd., Basingstoke, Hampshire, UK), pH 7.4 and incubated at 37 °C for 24 h. Enterobacteriaceae was confirmed with Nutrient Agar and Oxidase strip/stick according to NMKL 144, 2005.

4.2.3.6 Enumeration of *E. coli*

E. coli was enumerated in accordance with NMKL No. 125, 2005 on Tryptone Soya Agar (Oxoid CM 131, Oxoid Ltd., Basingstoke, Hampshire, UK), pH 7.3 using the pour plate method and overlaid with Violet Red Bile Agar (Oxoid CM 107), pH 7.4. The plates were incubated at 44 °C for 24 h and suspected colonies confirmed with EC Broth (Oxoid CM 853, Oxoid Ltd., Basingstoke, Hampshire, UK), pH 6.9, and Tryptone Water (Oxoid CM0087, Oxoid Ltd., Basingstoke, Hampshire, UK), pH 7.5. All plates were incubated at 44 °C for 24 h.

4.2.3.7 Enumeration of *Bacillus cereus*

Bacillus cereus was grown by spread plate on *Bacillus Cereus* Selective Agar (Oxoid CM 0617, Oxoid Ltd., Basingstoke, Hampshire, UK), pH 7.2 and confirmed on Blood Agar Base (Oxoid CM 55, Oxoid Ltd., Basingstoke, Hampshire, UK) at 30 °C for 24 h in accordance with NMKL No. 67, 2010.

4.2.3.8 Enumeration of *Staphylococcus aureus*

Staphylococcus aureus was enumerated by spread plate on Baird-Parker Agar (Oxoid CM 275, Oxoid Ltd., Basingstoke, Hampshire, UK), with Egg Yolk Tellurite Emulsion (SR54), pH 6.8 and incubated at 37 °C for 48 h according to NMKL No. 66, 2003. Suspected *Staphylococcus aureus* colonies were confirmed on Blood Agar Base (Oxoid CM 55, Oxoid Ltd., Basingstoke, Hampshire, UK) incubated at 37 °C for 48 h.

4.2.3.9 Detection of *Salmonella* spp

The presence of *Salmonella* spp in 25 g of sample was detected according to NMKL method No. 71, 1999. Twenty-five grams of the sample was pre-enriched in a non-selective medium, Buffered Peptone Water (Oxoid CM 0509, Oxoid Ltd., Basingstoke, Hampshire, UK), pH 7.2 incubated at 37 °C for 18 h. The pre-enriched sample was transferred into the selective medium, Rappaport-Vassiliadis Soy Peptone broth (Oxoid CM 0669, Oxoid Ltd., Basingstoke, Hampshire, UK), pH 5.4, which was incubated at 42 °C for 24 h. A loopful of the broth was then plated out on Xylose Lysine Desoxycholate Agar (Oxoid CM 0469, Oxoid Ltd., Basingstoke, Hampshire, UK), pH 7.4,

and incubated at 37 °C for 24 h. There were no suspected colonies to be verified biochemically and serologically.

4.2.3.10 Isolation of LAB and yeast

Ten colonies were selected from a segment of the highest dilution or appropriate MRS, M17 (for lactic acid bacteria) or Rose Bengal (for yeast) plate and streaked repeatedly on the appropriate agar plate until pure colonies were obtained.

4.2.3.11 Glycerol stock preparation of LAB and yeast

A pure single colony of lactic acid bacteria was inoculated into 20 ml of MRS broth in a bijou bottle and incubated overnight at 30 ºC. A mixture of 500 µL of 40 % glycerol (G/0650/08, Thermo Fisher Scientific, USA) and 500 µL of the overnight culture was pipetted into a 2 ml screw cap tube, vigorously shaken, and stored at - 80 °C. This procedure was used to store all the lactic acid bacteria isolates. The same procedure was used for storage of yeasts isolates except that they were grown overnight in Yeast Malt broth (Difco 271120, Bacton, Dickinson and Company Sparks, USA) and incubated overnight at 25 ºC.

4.2.3.12 Preparation of stocks for culture collection bank

1.5 ml of overnight broth cultures of LAB and yeasts were centrifuged for one min at 13,000 x *g* and the supernatant was discarded. The cells were re-suspended in 80 µL of 40 % glycerol and transferred to sterile Nunc screw cap tubes containing about 20 acid-washed sterile beads. They were mixed and frozen immediately on dry ice before transferring for storage at -80 °C in the Quadram Institute Culture Collection Bank.

4.2.4 Phenotypic characterisation of isolates

4.2.4.1 Colony morphology of bacteria isolates

Pure cultures of LAB on MRS plates were examined for colony colour and colony surface (smooth and shiny).

4.2.4.2 Catalase reaction of bacteria isolates

For each isolate, pure single bacteria colony was picked and emulsified on a clean glass slide containing a drop of 3 % hydrogen peroxide. Liberation of bubbles (free oxygen from the reaction) was interpreted as presence of catalase (Atter, 2012).

4.2.4.3 Oxidase test of bacteria isolates

A pure colony of LAB was smeared on oxidase identification strips (Oxoid Limited, Basingstoke, Hampshire, UK). A change in colour to purple indicated that the organism was catalase positive (Atter, 2012).

4.2.4.4 Gram staining of bacteria isolates

Gram staining was carried out using Gram staining kit (Remel, Thermo Fisher Scientific, USA). A loopful of the liquid culture was transferred to the surface of a clean glass slide and spread over a small area. The film was allowed to air-dry and fixed by passing it briefly through the Bunsen flame. The slide was flooded with crystal violet solution, Gram's iodine solution, 95 % alcohol and finally with Safranin (Wong, 2018) and examined under the microscope.

4.2.4.5 Microscopy of bacteria isolates

The cell morphology of the Gram-stained slides were examined under a phase contrast microscope (Olympus BX60F5, Japan).

4.2.4.6 Colony morphology of yeast isolates

The morphological characteristics considered for pure yeast colony included colour (pink, cream, white, off-white), surface (smooth, smooth and shiny, hirsute), appearance (elongated, ovoid, globose), elevation (raised, umbonate, concave), margine (entire, filiform or wavy) and size were examined for pure yeast colonies (Sulmiyati et al., 2019).

4.2.4.7 Growth pattern of yeast isolates in liquid medium

Growth patterns were also examined in 20 ml Yeast Mold broth, YM (BD 271120, Becton, Dickinson, USA) in bijou bottles. The characteristics examined included turbidity, gas production, sedimentation, growth on liquid media, pellicle formation between glass and liquid interphase (Sulmiyati et al., 2019).

4.2.4.8 Microscopy of yeast isolates

Cell morphology (shape, budding and arrangement) of pure yeast broth cultures were examined using a phase contrast microscope (Olympus BX60F5, Japan). A loopful of the broth culture was spread on a glass slides, a cover slip was placed on the glass slide and examined as wet mount under the microscope (X 40 objective lens).

4.2.4.9 Statistical analysis

Data analysis for pH and microbial population was done using analysis of variance (ANOVA) and Duncan test (SPSS version 21.0).

4.2.5 Molecular characterisation of isolates

4.2.5.1 16S rRNA gene colony PCR of pure bacteria isolates

Colony PCR was carried out to identify the bacterial isolates at species level. Processing of overnight liquid bacteria cultures for 16S PCR was done by pipetting 150 µL into an Eppendorf tube and centrifuged for 1' at 13,000 x *g*. Supernatant was removed, 150 µL ultra-pure water (UPH_2O) was added and vortexed to re-suspend. The suspension was centrifuged again, supernatant removed and re-suspended in 15 µL UPH_2O. Samples were heated in a thermal cycler (Biometra GmbH, Germany) at 95 °C 5'. Amplification of the PCR products were done using 16S AMP_F and AMP_R primers according to Baker et al., (2003). These were 16S AMP_F 5' GAG AGT TTG ATY CTGC GCT CAG 3' and AMP_R 5' AAG GAG GTG ATC CAR CCG CA 3' respectively. Amplification was performed in a 50 µL reaction volume consisting of 36.35 µL UP water, 0.25 µL GoTaq polymerase (Promega, USA), 0.4 µL of dNTP (25 mM each) Bioline, 1 µL (20 mM) forward primer, 1µL (20 mM) reverse primer, 10 µL 5X stabilizing buffer (WHITE), 1µL DNA template. The amplification of the mixture solution was conducted at 95 °C initial denaturation for 2 min, followed by 25 cycles of 95 °C denaturation for 30 sec., 55 °C annealing for 30 sec., 72 °C extension for 1min/kb, a final extension at 72 °C for 5 minutes (30 min for total extension time) and held at 4 °C. The PCR products were mixed with 5 µL of loading dye (B70245, Biolabs England) and run on 1 % agarose gel prepared with 5x Tris/Borate/EDTA (TBE) buffer.

The gel was loaded with 5 µL 1kb ladder (H1-314110, Bioline Hyperladder) and each of the PCR products and controls. Electrophoresis was run at 100 V for 1 h 20 m and visualised using a UV light (Alpha imager) (Wong, 2018).

4.2.5.2 Ribosomal internal transcribed spacer (ITS) colony PCR for yeast isolates

Processing of overnight liquid yeast cultures was carried out as described earlier for bacteria cultures using yeast specific primers. Samples were heated at 95 °C 5 min. The PCR reaction was performed with the primers (NL1) AMP_F 5'GCATATCAATAAGCGGAGGAAAA3' and (NL4) AMP_R 5'GGTCCGTGTTTCAAGACGG3'. The Amplification was performed in 50 µL reaction volume comprising 36.35 µL UP water, 0.25µL GoTaq polymerase, 0.4 µL of dNTP (25mM each) Bioline, 1µL (20mM) forward primer, 1µL (20mM) reverse primer, 10 µL 5X stabilizing buffer (WHITE) and 1µL DNA template. The amplification of the mixture solution was conducted using thermal cycler (Biometra GmbH, Germany). The cycling program was started with an initial denaturation at 94 °C for 5mins, followed by 25 cycles of 92 °C denaturation for 30 sec., 54 °C annealing for 30 sec., 72 °C extension for 1min/kb, final extension at 72 °C for 5 mins and held at 4 °C.

Following this, 1 % agarose (MB1200, Melford, UK) gel was prepared in 0.5 Tris/Borate/EDTA (TBE) buffer, 5 µL of loading dye (B70245, BioLabs England) added to each PCR product, 5 µL of 1kb ladder (H1-314110, Bioline Hyperladder). The PCR products were stained with ethidium bromide, electrophoresis run at 100 V for 1 h, and visualised under UV light using Alpha imager (Alpha Innotech). They were purified using QIAquick PCR purification kit (QIAGEN, Germany) following the manufacturers instruction (detailed description below). They were quantified and then prepared for sequencing with the forward primer NL1 (5'-

GCATATCAATAAGCGGAGGAAAA) and reverse primer, NL4 (5'-GGTCCGTGTTTCAAGACGG) and sent for Sanger sequencing at Eurofins, UK (Wong, 2018).

4.2.5.3 PCR purification

PCR products (bacteria and yeast) were purified using QIAquick PCR Purification Kit (QIAGEN, Germany) following the manufacturer's instruction. The purified PCR products were stored at -20 °C until used (Wong, 2018).

4.2.5.4 Rep-PCR reaction of LAB isolates

The LAB isolates were typed using Rep-PCR to select different isolates for whole genome sequencing as described by Owusu-Kwarteng et al., (2012) with slight modification. Rep-PCR reaction was performed using the primer GTG5 (5'- GTGGTGGTGGTGGTG -3'), Tm 7.2ºC, with GoTaq G2 (Promega, USA). Amplification was performed in a 50 µL reaction volume, consisting of 37.35 µL UPH_2O, 0.25µL GoTaq polymerase, 0.4µL of dNTP (25mM each) Bioline, 10 µL 5X stabilizing buffer (WHITE), 1µL DNA template. The amplification was conducted using thermal cycler (Biometra GmbH, Germany) programmed at 94 °C initial denaturation for 4mins, followed by 30 cycles of 94 °C denaturation for 30sec., 45 °C annealing for 1min., 72 °C extension for 8 min and final extension at 72 °C for 16 minutes.

Following this, 1.6 % agarose gel was prepared in 0.5 Tris/Borate/EDTA (TBE) buffer, 5 µL of loading dye (B70245, Brolabs England) added to each PCR product, 5 µL of 1 kb ladder (H1-314110, Bioline Hyperladder) as reference and PCR products and controls loaded. Electrophoresis was run at 115 V for 5 h 30 min. The gels were then stained in ethidium bromide for 30 m and

rinsed in deionised water for 1 m after which they were visualised under UV light and images captured using Alpha imager (Alpha Innotech) (Wong, 2018).

4.2.5.5 Genomic DNA extraction from LAB

Genomic DNA extraction was performed according to the method described by Foster-Nyarko et al., (2020) with the following modifications. The DNA of pure LAB isolates was extracted from 50 µl of resuspended overnight cultures, using a 96 well plate DNA extraction method. In each plate well, containing 50 µL of the cell suspension, 100 µL of lysing buffer (0.02 g lysozyme, 10 mls of TE buffer, 100 µL RNAse A of 10 mg/ml and 100 µL and Mutanolysin (10 KU/ml)) were added. Adhesive seal was firmly attached to cover the wells and placed on a thermomixer set to 37 °C and shaked at 1600 rpm for 30 min. The plate was centrifuged briefly at maximum speed (5810R, Eppendorf, Germany) braced with hard-shell and skirted plate to avoid cross contamination. 10 µL of lysing additive (528 µL TE buffer, 600 µL 10 % SDS buffer, 60 µL Proteinase K and 12 µL RNAse A) were added to each well and re-suspended. The wells were sealed firmly with adhesive seal and placed again on thermomixer set to 65 °C 1600 rpm for 15 min. The plate was briefly centrifuged again to avoid crossed contamination. About 100 µL of the suspension was pipetted from the wells to a new lo-bind PCR 96 well plate for DNA purification using solid-phase reversible immobilisation magnetic beads (AMPure XP, Beckman Coulter Inc, USA). 50 µL of the magnetic beads were added to each well, mixed by pipetting and incubated at room temperature for 5 min. Plates were placed on a magnet instrument and left for 5 min till it settled. The supernatant was removed and the beads were washed with 100 µL of freshly prepared 80 % ethanol by running the liquid over the magnetic beads. Ethanol was removed and the wash repeated for two more times. The plate was allowed to dry off for 2 min, taken off the magnetic

apparatus. The DNA was eluted from the beads by addition of 50 µL 10 mM Tris-Cl, mixed by pipetting and incubated at room temperature for 5 min. The plate was placed back on the magnetic rack for 2 min, and 50 µL of the genomic extraction were transferred into a new lo-bind 96 well PCR plate. DNA concentration was quantified and stored at -20 °C until ready for sequencing.

4.2.5.6 DNA quantification

DNA concentrations were measured according to the method described ealier in chapter 3 (3.2.3.3) (Atter et al., 2021).

4.2.5.7 Whole genome sequencing of LAB isolates

Whole genome sequencing of the LAB isolates was conducted at the Earlham Institute (Norwich, UK). The gDNA extracted from pure cultures was used to construct low input transposase enabled (LITE) libraries. Libraries were sequenced using the Illumina HiSeq4000 platform with a 150bp paired end read.

4.2.6 Bioinformatic analysis

4.2.6.1 Sanger sequences analysis

Sequencing read sets from the yeast isolates were assembled and manually revised using EditSeq v 5.06 and SeqMan II v 5.06 software packages (DNASTAR. Inc). The cleaned and assembled sequences were identified using RDP database using typed strains only. The sequences were identified to the species level with percentage identity of the sequence similarities from 99 - 100 % to those in the database.

4.2.6.2 Genome assembly of LAB isolates

4.2.6.2.1 Cleaning of contaminated samples

Read sets resulting from Illumina whole genome sequencing were subjected to taxonomic classification against the NCBI database with centrifuge from contamination using centrifuge v 1.0.3 (https://ccb.jhu.edu/software/centrifuge). Classified reads were then filtered with kt extract, contained in the ktoolu software package (https://github.com/cschu/ktoolu). Reads that were classified as fungal were discarded.

4.2.6.2.2 Adapter removal, quality control and read normalization

Adapters were removed from the 3'and 5'-end, bases with quality less than phred 3 were removed from both ends and reads with a length below 100bp or an average quality of less than phred 20 were discarded using the bbduk tool from the software package bbmap v 37.24 (https://jgi.doe.gov/data-and-tools/bbtools). Cleaned read sets were normalized to 2x-100x coverage with bbnorm (bbmap v 37.24).

4.2.6.2.3 Assembly

The quality controlled and normalized reads were assembled utilising the spades-optimizing mode of the unicycler-pipeline (unicycler: 0.4.3_cs2, spades: 3.8.1) (https://github.com/rrwick/Unicycler). For the optimization, sample-specific k-mer ranges were determined by unicycler. As part of the pipeline, reads were error-corrected by spades and the resulting contigs polished with pilon v 1.22.

4.2.6.2.4 Assembly quality assessment

Assemblies were quality checked with QUAST v 4.3 http://quast.sourceforge.net/quast) and BUSCO v 3.0 (evaluates orthologous gene completeness) (http://busco.ezlab.org/). Assembled scaffolds/contigs were taxonomically classified with blobtools v 0.9.19 (https://blobtools.readme.io/docs).

4.2.6.2.5 Genome annotation

Assemblies were submitted to microbial genome annotation using prokka v 1.12 (https://github.com/tseemann/prokka).

4.2.6.3 Phylogeny

The sequence genome of all the LAB isolates were subjected to analysis using Molecular Evolutionary Genetics Analysis (MEGA) version 6 software to determine their phylogenetic relatedness.

4.3 Results

4.3.1 Changes in pH during *Hausa koko* production

The pH of samples as taken at different stages during the production of *Hausa koko* by traditional food processors in 5 different regions of Ghana is presented in Figure 11. The samples were from Tamale Dabokpe (TAD), Sunyani (SUN), Mankessim (MAN), Dodowa (DOD) and Accra Madina Zongo (AMZ) and came from a total of 12 production sites. The pH of the raw material, millet grains ranged from 6.02 ± 0.01 to 6.53 ± 0.01. When the grains were cleaned washed and then steeped in water and allowed to ferment for 12 h during steeping, the pH of the samples reduced drastically to between 4.08 ± 0.01 and 4.59 ± 0.01. At 24 hours of steeping the pH values recorded ranged from 4.28 ± 0.01 to 4.34 ± 0.01. At this stage the processors recover the steeped millet grains by decanting off the water, spices are added to the grains and milled together. The pH values recorded for milled millet and spices from the different production sited ranged from 3.91 ± 0.01 to 4.42 ± 0.01. The processors then add water to the milled millet and spices and mix it into a slurry and allow it to ferment for about 48 hours. During fermentation the slurry separates into a sediment at the bottom and a supernatant on top. The pH values of supernatants collected from the various production sites ranged from 3.27 ± 0.01 to 3.68 ± 0.01 and that of the sediments between 3.28 ± 0.01 and 3.65 ± 0.01. The pH of *Hausa koko* samples from the production sites were in the range of 3.51 ± 0.01 to 3.99 ± 0.01. With the exception of supernatant and sediment samples from Tamale and Accra, there were significant differences in the pH values at the different stages of processing ($p \leq 0.05$).

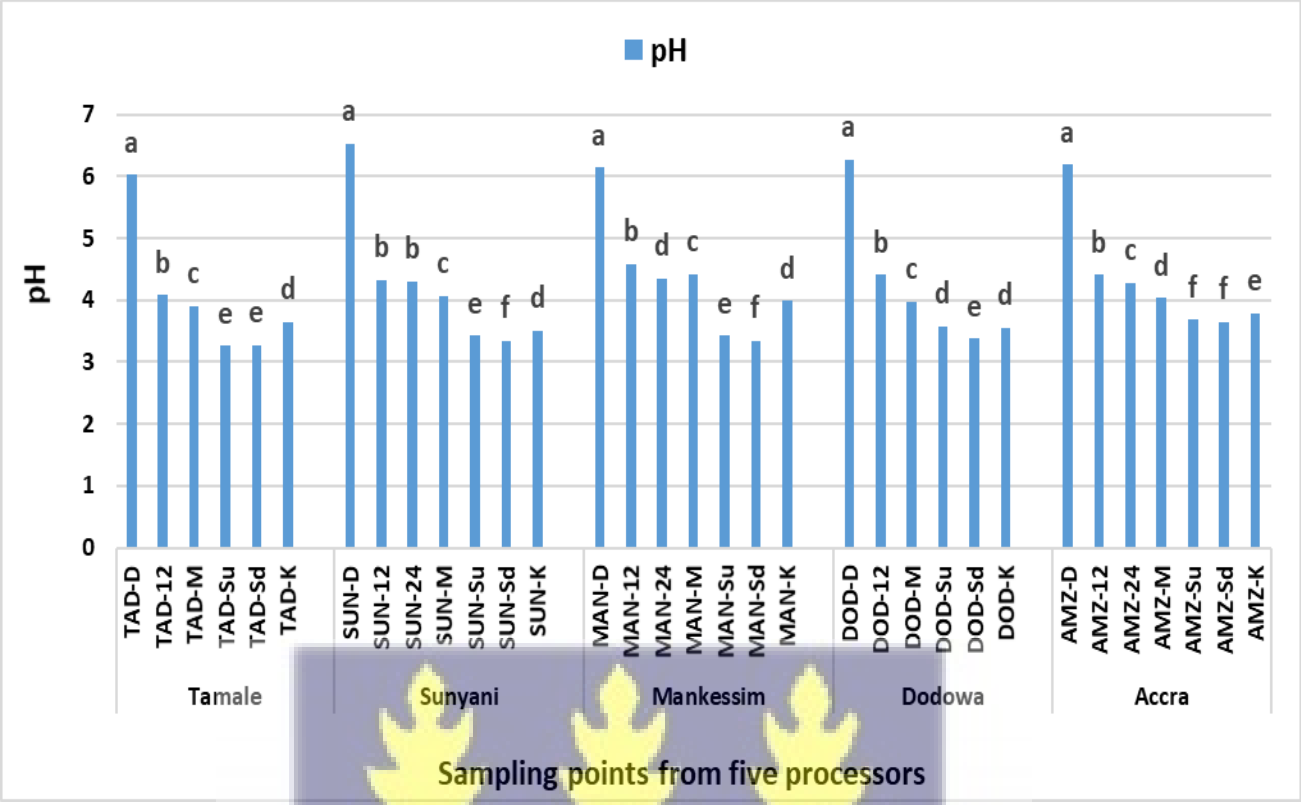


Figure 11: pH values at various stages of *Hausa koko* production from five processors

NB:

Sampling points:

D = dry millet grains; 12h = 12 h steeped millet; 24h = 24 h steeped millet; M = milled millet with spices; Su = supernatant of fermented slurry; Sd = sediment of fermented slurry; K = *Hausa koko*

Sampling sites:

Tamale Dabokpa (TAD); Sunyani (SUN); Mankessim (MAN); Dodowa (DOD); Accra Madina Zongo (AMZ)

a - f = bars with different letters are significantly different at $P \leq 0.05$

4.3.2 Population of fermentative and other microorganisms at different stages in the production of *Hausa koko*

The population of fermentative microorganisms in the samples collected at various stages in the production of *Hausa koko* from the 5 production sites in 5 different regions of Ghana is shown in Figure 12. These were lactic acid bacteria and yeasts. The population of *Lactococci* which are also LAB was determined separately on M17 to see if they played a key role in the fermentation of the millet product. The LAB counts in the dried grains were in the range of log 10 3.18 ± 0.01 to 4.79 ± 0.01 CFU/g. At the end of the slurry fermentation the LAB population had increased by four log units to between log 7.64 ± 0.02 and log 8.94 ± 0.01 CFU/g. In the samples of cooked *Hausa koko* the LAB population was in the range of log 2.77 ± 0.02 to log 3.95 ± 0.01 CFU/g. The corresponding yeasts populations were log 2.02 ± 0.03 to log 3.88 ± 0.01 CFU/g in the millet grains, log 4.54 ± 0.02 to log 6.98 ± 0.01 CFU/g at the end of slurry fermentation, and log 2.10 ± 0.02 to log 2.98 ± 0.01 CFU/g in the *Hausa koko* samples. The population of *Lactococci* which were enumerated in the samples were usually about half of the counts recorded for the LAB which were enumerated on MRS, though in a few instances they were much higher (Figure 15a). Generally, there were significant differences ($p \leq 0.05$) in the LAB and yeast counts at the different stages of processing.

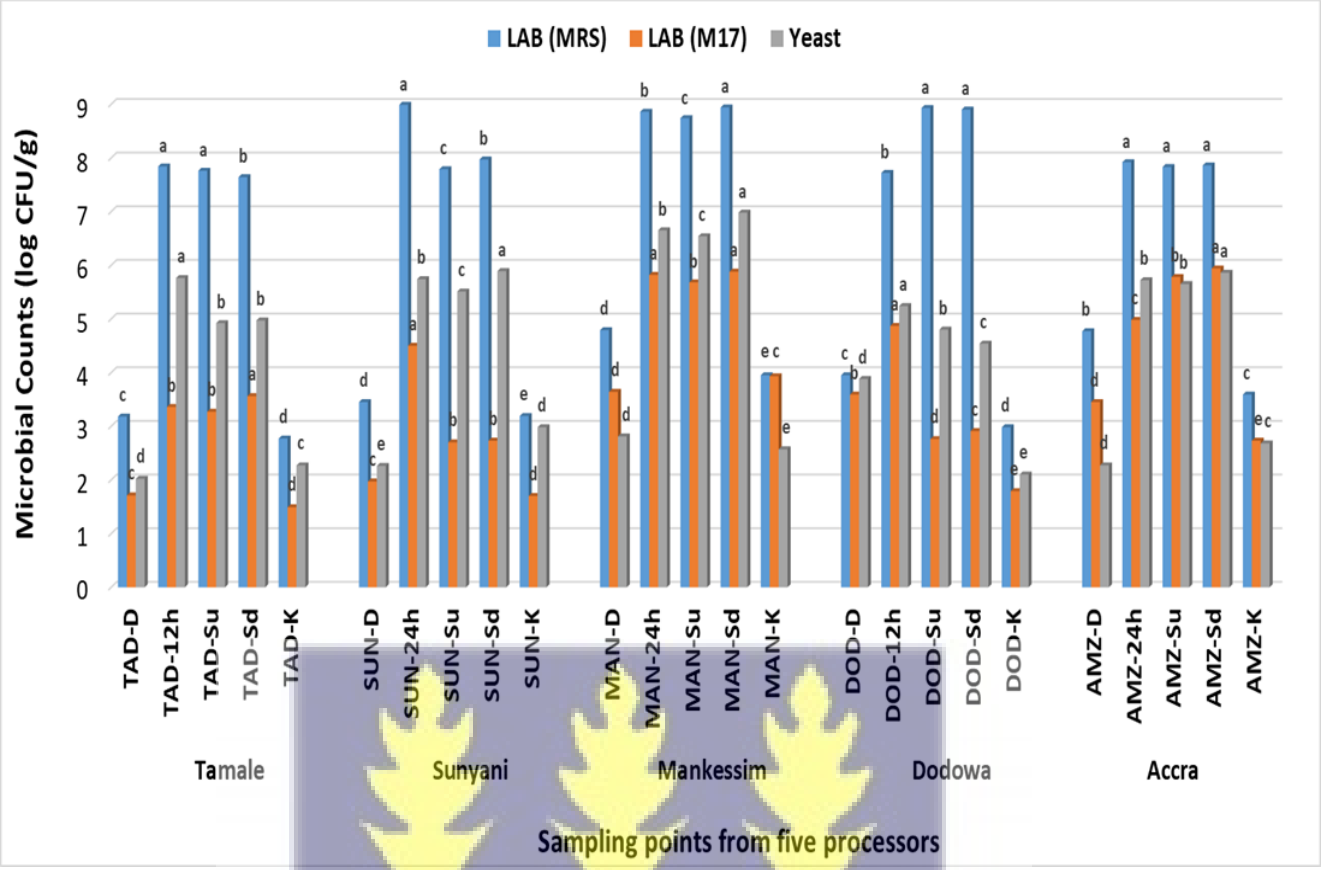


Figure 12: LAB and yeast population in log CFU/g at various stages of *Hausa koko* production from five processors.

NB:

Sampling points:

D = dry millet grains; 12h = 12 h steeped millet; 24h = 24 h steeped millet; M = milled millet with spices; Su = supernatant of fermented slurry; Sd = sediment of fermented slurry; K = *Hausa koko*

Sampling sites:

Tamale Dabokpa (TAD); Sunyani (SUN); Mankessim (MAN); Dodowa (DOD); Accra Madina Zongo (AMZ)

a - e = bars with different letters are significantly different at $P \leq 0.05$

Generally, there were significant differences ($p \leq 0.05$) in the microbial population at the different stages of processing among the different processors. The population of aerobic mesophiles, Enterobacteriaceae, *S. aureus, E. coli, B. cereus* and *Salmonella* spp from the five production sites are shown in Figure 13. As expected, aerobic mesophilic counts were generally high, ranging from log 4.74 ± 0.04 to log 6.85 ± 0.05 CFU/g in the dried millet grains. They increased during fermentation by three to four log units to between log 8.80 ± 0.02 to log 9.98 ± 0.01 CFU/g in the sediments. Cooking of the *Hausa koko* resulted in a reduction in the population of aerobic mesophiles by about 5 log units to between log 3.03 ± 0.01 and log 4.78 ± 0.02 CFU/g in the cooked *Hausa koko* samples.

The population of Enterobacteriaeceae ranged from log 2.49 ± 0.04 to log 6.72 ± 0.04 CFU/g in the dry millet grains but in cooked ready to eat *Hausa koko* samples, they were either not detected or present at not more than log 2.41 ± 0.13 CFU/g. *Staphylococcus aureus* was not detected in any of the cooked ready to eat *Hausa koko* samples but were present at the early production stages in some of the samples. With regards to *Bacillus cereus*, the bacterium was not detected in any of the samples from the production site at Takoradi (TAD). At the production site in Mankessim (MAN), *B. cereus* was not detected after slurry fermentation, whilst at Dodowa it was present after the slurry fermentation but eliminated in the ready to eat *Hausa koko*. At the production sites in Sunyani (SUN) and Accra (AMZ) *B. cereus* occurred throughout processing and was also found in the cooked ready to eat *Hausa koko* samples at concentrations of log 1.23 ± 0.12 and 1.04 ± 0.05 CFU/g. With the exception of one processor who recorded *E. coli* at all the stages of production, the others recorded *E. coli* counts up to the end of steeping of the millet grains or after the steeped grains had been milled together with spices, but not in the fermented slurries. In the samples from Mankessim in which the *E. coli* was present in the ready to eat *Hausa koko* sample,

the *E. coli* count was log 1.10 ± 0.02 CFU/g. *Salmonella* spp was not present in any of the samples analysed from the five production sites at any stage of production, nor in the cooked *Hausa koko.*

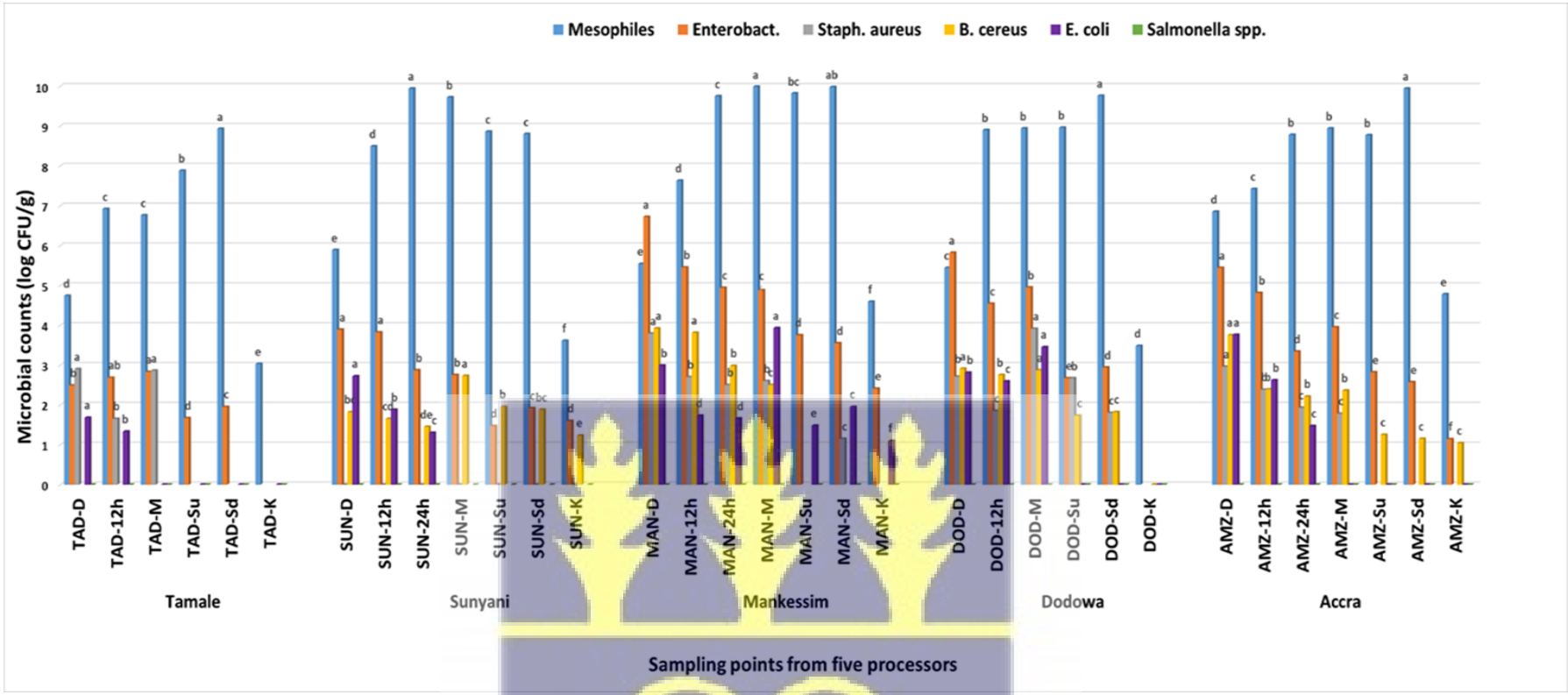


Figure 13: Microbial population in log CFU/g at various stages of *Hausa koko* production from five processors

NB:

Sampling points: D = dry millet grains; 12h = 12 h steeped millet; 24h = 24 h steeped millet; M = milled millet with spices; Su = supernatant of fermented slurry; Sd = sediment of fermented slurry; K = *Hausa koko*

Sampling sites: Tamale Dabokpa (TAD); Sunyani (SUN); Mankessim (MAN); Dodowa (DOD); Accra Madina Zongo (AMZ)

a – f = bars with different letters are significantly different at $P \leq 0.05$

4.3.3. Characterisation and identification of lactic acid bacteria

Isolates on MRS and M17 agar which were Gram-positive, catalase negative, and oxidase negative were assumed to be LAB. They were mostly rods which occurred in singles, pairs or chains. They were further characterised by bacteria colony PCR, profiled and differentiated using (GTG)5 based rep-PCR as shown in Figure 14.

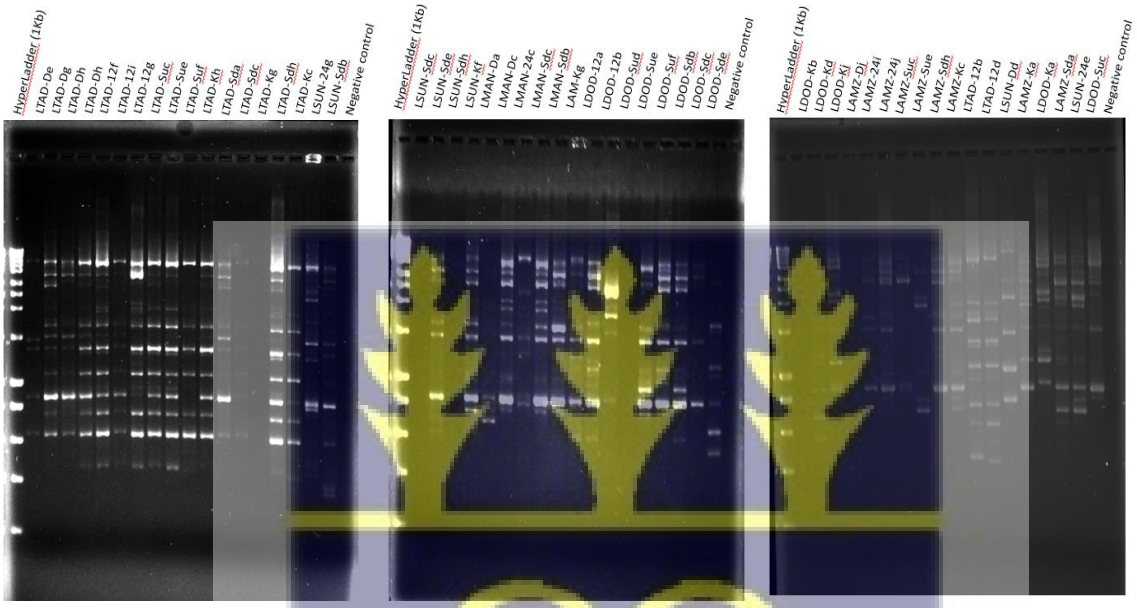


Figure 14: Gel images of rep-PCR of some LAB isolates. Lane 1; 1 kb hyperladder, lanes 2-19; LAB isolates; lane 20 negative control.

A total of 95 of the LAB isolates that appeared different by observation of the gel images of rep-PCR were selected from the initial 500 LAB isolates and analysed by whole genome sequencing. Ninety (90) of these isolates were successfully sequenced, but 5 were either contaminated or had poor reads. Out of the total 90 isolates sequenced successfully, 28 were short rods which occurred in singles, pairs or chains and identified as *Limosilactobacillus pontis*. *L. pontis* was the most dominant of the LAB and represented 31.11 % of the LAB population. The second dominant LAB were, 15 each in number. One was coccoid and occurred in pairs or tetrads representing 16.67 %

of the LAB population and were identified as *Pediococcus acidilactici* whilst the other also representing 16.67 % were short rods and occurred in singles, pairs or chains and were identified as *Limosilactobacillus fermentum*. The third dominant LAB, 10 in number representing 11.11 % of the isolates were coccoids which occurred in pairs and tetrads were identified as *Pediococcus pentosaceus.* The next dominant LAB, 9 in number which were short rods and which occurred in singles, pairs and chains were identified as *Limosilactobacillus reuteri*, representing 10 % of the LAB isolates. Six (6) short rods which occurred in singles, pairs and chains representing 6.67 % were identified as *Weissella confusa*. Three (3) isolates identified as *Schleiferilactobacillus harbinensis* were mainly long rods in singles representing 3.33 %. The rest were 2 each in number representing 2.22 % each which were identified as *Lactiplantibacillus plantarum* (short rod shaped in singles, pairs and chains), and *Lacticaseibacillus paracasei* (rods mainly in singles). All the isolates identified with their accession numbers and their similarity to the closest relative identified using blast in the National Center for Biotechnology Information (NCBI) database are presented in Table 3.

Table 3: Identified LAB from five (5) different production sites

Site/Product	Isolate	Description	% identity	Accession No
Tamale/Grains	LTAD-De	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.18	CP045530.1
Tamale/Grains	LTAD-Dh	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.18	CP045530.1
Tamale/12 h	LTAD-12i	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.31	CP045530.1
Tamale/Supernatant	LTAD-Suc	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.02	CP045530.1
Tamale/Supernatant	LTAD-Sue	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	98.86	CP045530.1
Tamale/*H.koko*	LTAD-Kh	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.17	CP045530.1
Tamale/Supernatant	LTAD-Suf	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.24	CP045530.1
Tamale/12 h	LTAD-12b	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.4	CP045530.1
Tamale/*H.koko*	LTAD-Kg	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.17	CP045530.1
Tamale/Sediment	LTAD-Sdh	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.18	CP045530.1
Tamale/*H.koko*	LTAD-Kc	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.24	CP045530.1
Accra/24 h	LAMZ-24a	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.47	CP045530.1
Accra/24 h	LAMZ-24b	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.47	CP045530.1
Tamale/12 h	LTAD-12e	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.17	CP045530.1
Tamale/Grains	LTAD-Dg	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.18	CP045530.1
Accra/24 h	LAMZ- 24d	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.18	CP045530.1
Accra/24 h	LAMZ-24f	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.17	CP045530.1
Accra/24 h	LAMZ-24h	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.47	CP045530.1
Tamale/12 h	LTAD-12d	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.24	CP045530.1
Tamale/Sediment	LTAD-Sdg	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.18	CP045530.1
Dodowa/*H.koko*	CDOD-Kg	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.19	CP045530.1
Accra/Grains	CAMZ-De	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.4	CP045530.1

Accra/Sediment	LAMZ-Sdb	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.47	CP045530.1
Accra/Sediment	LAMZ-Sdi	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.3	CP045530.1
Tamale/Supernatant	LTAD-Sua	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.24	CP045530.1
Accra/Sediment	LAMZ-Sdc	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.26	CP045530.1
Tamale/12 h	LTAD-12f	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.18	CP045530.1
Tamale/12 h	LTAD-12g	*Limosilactobacillus pontis* strain LP475 chromosome, complete genome	99.4	CP045530.1
Tamale/Sediment	LTAD-Sda	*Limosilactobacillus fermentum* strain SRCM 103290 chromosome, complete genome	99.23	CP035055.1
Mankessim/Grains	LMAN-Dc	*Limosilactobacillus fermentum* strain SRCM 103290 chromosome, complete genome	99.42	CP035055.1
Mankessim/Sediment	LMAN-Sdc	*Limosilactobacillus fermentum* strain SRCM 103290 chromosome, complete genome	99.42	CP035055.1
Mankessim/Sediment	LMAN-Sdb	*Limosilactobacillus fermentum* strain SRCM 103290 chromosome, complete genome	99.37	CP035055.1
Dodowa/Supernatant	LDOD-Sue	*Limosilactobacillus fermentum* strain IMDO 130101 genome assembly, chromosome:I	99.31	LT906621.1
Dodowa/Supernatant	LDOD-Suf	*Limosilactobacillus fermentum* strain SRCM 103290 chromosome, complete genome	99.46	CP035055.1
Dodowa/Sediment	LDOD-Sdb	*Limosilactobacillus fermentum* strain SRCM 103290 chromosome, complete genome	99.32	CP035055.1
Accra/24 h	LAMZ-24j	*Limosilactobacillus fermentum* strain SRCM 103290 chromosome, complete genome	99.7	CP035055.1
Accra/Grains	LAMZ-Dj	*Limosilactobacillus fermentum* strain IMDO 130101genome assembly, chromosome:I	99.39	LT906621.1
Accra/Sediment	LAMZ-Sdh	*Limosilactobacillus fermentum* strain SRCM 103290 chromosome, complete genome	99.23	CP035055.1
Accra/*H.koko*	LAMZ-Ka	*Limosilactobacillus fermentum* strain SRCM 103290 chromosome, complete genome	99.23	CP035055.1
Dodowa/Supernatant	LDOD-Suc	*Limosilactobacillus fermentum* strain SRCM 103290 chromosome, complete genome	99.42	CP035055.1
Mankessim/Sediment	CMAN-Sdg	*Limosilactobacillus fermentum* strain IMDO 130101 genome assembly,chromosome:I	99.31	LT906621.1
Dodowa/12 h	LDOD-12b	*Limosilactobacillus fermentum* strain SRCM 103290 chromosome, complete genome	98.23	CP035055.1
Dodowa/Sediment	LDOD-Sdc	*Limosilactobacillus fermentum* strain IMDO 130101 genome assembly,chromosome:I	99.47	LT906621.1
Sunyani/Sediment	LSUN-Sdh	*Pediococcus acidilactici* strain BCC1 chromosome, complete genome	90.18	CP018763.1
Sunyani/*H.koko*	LSUN-Kf	*Pediococcus acidilactici* strain SRCM102732 chromosome, complete genome	99.97	CP028249.1
Accra/*H.koko*	LAMZ-Kc	*Pediococcus acidilactici* strain CACC 537 chromosome, complete genome	91.8	CP048019.1

Accra/Grains	LAMZ-De	*Pediococcus acidilactici* strain CACC 537 chromosome, complete genome	91.87	CP048019.1
Accra/Grains	LAMZ-Dg	*Pediococcus acidilactici* strain FDAARGOS_1007 chromosome, complete genome	94.66	CP066046.1
Accra/Grains	LAMZ-Da	*Pediococcus acidilactici* strain PMC65 chromosome, complete genome	93.66	CP053421.1
Accra/Grains	LAMZ_Dc	*Pediococcus acidilactici* strain PMC65 chromosome, complete genome	96.59	CP053421.1
Dodowa/Sediment	LDOD-Sda	*Pediococcus acidilactici* strain HN9 chromosome, complete genome	94.13	CP061715.1
Mankessim/*H.koko*	LMAN-Kb	*Pediococcus acidilactici* strain PMC65 chromosome, complete genome	96.59	CP053421.1
Mankessim/*H.koko*	LMAN-Kh	*Pediococcus acidilactici* strain PMC202 chromosome, complete genome	96.59	CP080397.1
Tamale/Sediment	LTAD-Sdj	*Pediococcus acidilactici* strain SRCM101189 chromosome, complete genome	99.25	CP021529.1
Mankessim/Supernatant	LMAN-Sua	*Pediococcus acidilactici* strain PMC48 chromosome, complete genome	93.67	CP050079.1
Mankessim/Sediment	LMAN-Sdi	*Pediococcus acidilactici* strain CACC 537 chromosome, complete genome	91.87	CP048019.1
Mankessim/Sediment	LMAN-Sda	*Pediococcus acidilactici* strain HN9 chromosome, complete genome	92.27	CP061715.1
Sunyani/Grains	LSUN-Dd	*Pediococcus acidilactici* strain SRCM101189 chromosome, complete genome	99.31	CP021529.1
Accra/24 h	LAMZ-24i	*Pediococcus pentosaceus* strain JQI-7 chromosome, complete genome	99.93	CP023655.1
Sunyani/24 h	LSUN-24e	*Pediococcus pentosaceus* strain SRCM100194 chromosome, complete genome	99.95	CP021927.1
Dodowa/Sediment	CDOD-Sdd	*Pediococcus pentosaceus* strain FDAARGOS_1009 chromosome, complete genome	99.93	CP066043.1
Mankessim/Supernatant	CMAN-Suc	*Pediococcus pentosaceus* strain FDAARGOS_1009 chromosome, complete genome	99.79	CP066043.1
Dodowa/Supernatant	CDOD-Sua	*Pediococcus pentosaceus* strain SRCM102734 chromosome, complete genome	99.94	CP028254.1
Dodowa/*H.koko*	CDOD-Kh	*Pediococcus pentosaceus* strain SRCM102734 chromosome, complete genome	99.92	CP028254.1
Dodowa/*H.koko*	CDOD-Kf	*Pediococcus pentosaceus* strain JQI-7 chromosome, complete genome	99.43	CP023655.1
Accra/24 h	CAMZ-24c	*Pediococcus pentosaceus* strain SL001 chromosome, complete genome	99.61	CP039378.1
Mankessim/Grains	LMAN-Df	*Pediococcus pentosaceus* strain JQI-7 chromosome, complete genome	99.45	CP023655.1
Mankessim/24 h	LMAN-24g	*Pediococcus pentosaceus* strain KCCM 40703 chromosome, complete genome	99.12	CP020018.1
Sunyani/Sediment	LSUN-Sdc	*Limosilactobacillus reuteri* strain ATG-F4 chromosome, complete genome	99.05	CP035790.1
Accra/Supernatant	LAMZ-Suc	*Limosilactobacillus reuteri* strain ATG-F4 chromosome, complete genome	98.09	CP035790.1

Accra/Sediment	LAMZ-Sda	*Limosilactobacillus reuteri* strain reuteri chromosome, complete genome	98.89	CP045049.1
Mankessim/Grains	LMAN-Di	*Limosilactobacillus reuteri* strain IRT, complete genome	98.88	CP011024.1
Sunyani/24 h	LSUN-24g	*Limosilactobacillus reuteri* strain ATG-F4 chromosome, complete genome	99.05	CP035790.1
Sunyani/Sediment	LSUN-Sde	*Limosilactobacillus reuteri* strain YLR001 chromosome, complete genome	98.8	CP065540.1
Mankessim/24 h	LMAN-24c	*Limosilactobacillus reuteri* strain YLR001 chromosome, complete genome	98.8	CP065540.1
Accra/*H.koko*	LAMZ-Kg	*Limosilactobacillus reuteri* strain ATG-F4 chromosome, complete genome	98.56	CP035790.1
Dodowa/Supernatant	LDOD-Sud	*Limosilactobacillus reuteri* strain TK-F8A chromosome, complete genome	99.05	CP045605.1
Sunyani/Sediment	LSUN-Sdb	*Weissella confusa* strain N17 chromosome, complete genome	98.46	CP049097.1
Tamale/Sediment	LTAD-Sdc	*Weissella confusa* strain LM1 chromosome, complete genome	98.78	CP080582.1
Sunyani/24 h	LSUN-24d	*Weissella confusa* strain VTT E-133279 chromosome, complete genome	98.73	CP027563.1
Sunyani/Supernatant	LSUN-Sui	*Weissella confusa* strain VTT E-133279 chromosome, complete genome	98.09	CP027563.1
Sunyani/Supernatant	LSUN-Suh	*Weissella confusa* strain N17 chromosome, complete genome	99.1	CP049097.1
Mankessim/Supernatant	LMAN-Sue	*Weissella confusa* strain LM1 chromosome, complete genome	99.06	CP080582.1
Dodowa/Sediment	LDOD-Sde	*Schleiferilactobacillus harbinensis* strain M1 chromosome, complete genome	98.93	CP045143.1
Dodowa/Sediment	LDOD-Sdj	*Schleiferilactobacillus harbinensis* strain M1 chromosome, complete genome	98.7	CP045143.1
Dodowa/*H.koko*	LDOD-Kd	*Schleiferilactobacillus harbinensis* strain LH991 chromosome	99.35	CP045180.1
Dodowa/*H.koko*	LDOD-Kb	*Lactiplantibacillus plantarum* strain ZS2058, complete genome	99.46	CP012343.1
Dodowa/*H.koko*	LDOD-Ka	*Lactiplantibacillus plantarum* strain ZS2058, complete genome	99.46	CP012343.1
Tamale/Sediment	LTAD-Sdb	*Lacticaseibacillus paracasei* strain IIA, complete genome	99.95	CP014985.1
Accra/Supernatant	CAMZ-Sua	*Lacticaseibacillus paracasei* strain Lp02 chromosome, complete genome	99.97	CP039707.1

4.3.4 Phylogeny

The constructed phylogenetic diagram based on whole genome sequence results which depicts the relatedness of the different species of LAB isolated from the different production sites in the production of *Hausa koko* in terms of their differences and similarities is presented in Figure 15. As expected, related species clustered together with a few others towing a different trend where different species related in certain features also clustered together.

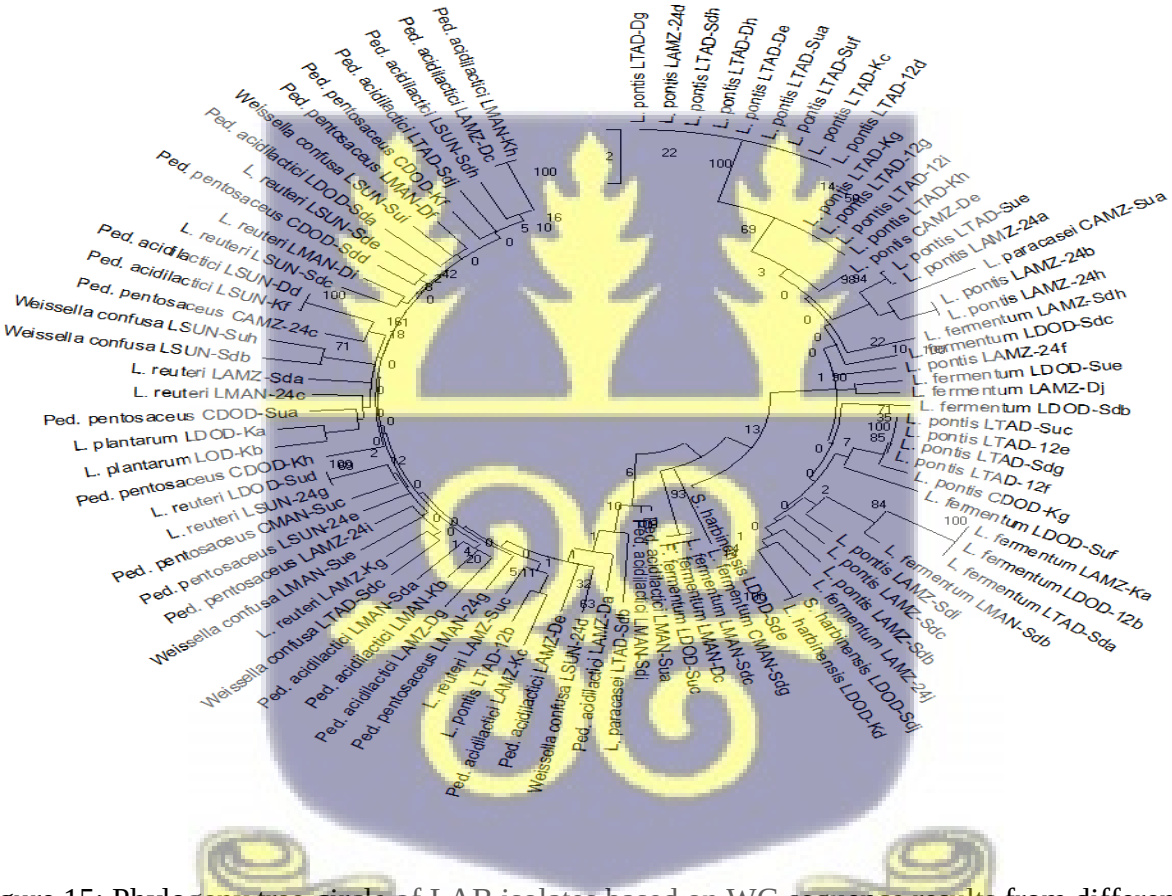


Figure 15: Phylogeny tree-circle of LAB isolates based on WG sequence results from different stages in the traditional processing of millet into *Hausa koko* from five production sites in different regions of Ghana.

4.3.5 The proportion of different lactic acid bacteria species in the total population of LAB occurring in the production of *Hausa koko*

The percentage of different species in the total number of lactic acid bacteria isolated at the different production sites are shown in Table 4. The total number which constitutes 100 %, represents all the LAB isolated at the different stages in the production of the *Hausa koko* at the production sites. The most diverse number of LAB species based on whole genome sequencing, numbering 7, were isolated at the Dodowa production site, with *L. fermentum* as the predominant specie. *P. acidilactici* was found in all five production sites. *L. fermentum, L. reuteri* and *P. pentosaceus* were found in four out of the five production sites. *L. pontis* and *W. confusa* were found in three out of the five production sites and *L. paracasei* was isolated at two out of five production sites. These isolates differed in where (processing stages) they were present or absent at the different processing sites in the various regions of Ghana.

Table 4. Percentage (%) of special differences of the lactic acid bacteria population at the different production sites

Lactic acid bacteria	***Hausa koko* production site**				
	Dodowa	**Tamale**	**Sunyani**	**Mankessim**	**Accra**
L. fermentum	33.33	4.55	-	26.67	16.67
L. reuteri	5.56	-	27.27	13.33	12.50
W. confusa	-	4.55	36.37	6.67	-
P. acidilacti	5..56	4.55	27.27	33.33	20.83
L. pontis	5.56	81.80	-	-	37.50
P. pentosaceus	22.22	-	9.09	20.00	8.33
L. paracasei	-	4.55	-	-	4.17
L. plantarum	11.11	-	-	-	-
S. harbinensis	16.66	-	-	-	-

4.3.6 The composition of lactic acid bacteria at different stages of *Hausa koko* production

The composition of the lactic acid bacteria population at different stages of *Hausa koko* production i.e. dry millet grains, during millet fermentation (12 and 24 h), supernatant, sediment and *Hausa koko* for the five production sites as a unit is presented in Table 5. The LAB population on the millet grains was dominated by *P. acidilactici*. The isolates from the 12 and 24 h of fermentation samples were dominated by *L. pontis*. In the supernatants *L. pontis* dominated whilst in the sediments *L. fermentum* was dominant. In the *Hausa koko* samples *P. acidilactici* and *L. pontis* were the dominant LAB. *L. fermentum, L. pontis, L. reuteri* and *P. pentosaceus* were the only LAB species which were isolated at all the different stages of *Hausa koko* production processs, though they were not isolated at all the production sites. The most varieties of LAB species occurred in the sediments of the fermenting slurries i.e. 8 different species.

Table 5: Composition of LAB population at different stages of *Hausa koko* production at the five production sites

Processing Stages	**Isolates**	**Percentage (%) Occurrence**
Dry Grains	***Limosilactobacillus pontis***	30.77
13 Isolates, 5 strains	***Limosilactobacillus fermentum***	15.38
	Pediococcus acidilactici	38.46
	Limosilactobacillus reuteri	7.69
	Pediococcus pentosaceus	7.69
12 & 24 h	***Limosilactobacillus pontis***	55.00
20 Isolates, 5 strains	***Limosilactobacillus fermentum***	10.00
	Weissella confusa	5.00
	Limosilactobacillus reuteri	10.00
	Pediococcus pentosaceus	20.00
Supernatant	***Limosilactobacillus pontis***	25.00
16 Isolates, 7 strains	***Limosilactobacillus fermentum***	18.75

	Limosilactobacillus reuteri	12.50
	Lacticaseibacillus paracasei	6.25
	Pediococcus pentosaceus	12.50
	Weissella confusa	18.75
	Pediococcus acidilactici	6.25
Sediment	***Limosilactobacillus fermentum***	26.92
27 Isolates, 8 strains	***Limosilactobacillus pontis***	19.23
	Weissella confusa	7.69
	Limosilactobacillus reuteri	11.54
	Pediococcus acidilactici	19.23
	Schleiferilactobacillus harbinensis	7.69
	Lacticaseibacillus paracasei	3.85
	Pediococcus pentosaceus	3.85
Hausa koko	***Limosilactobacillus pontis***	26.67
15 Isolates, 7 strains	*Pediococcus acidilactici*	26.67
	Lactiplantibacillus plantarum	13.33
	Pediococcus pentosaceus	13.33
	Limosilactobacillus fermentum	6.67
	Schleiferilacctobacillus harbinensis	6.67
	Limosilactobacillus reuteri	6.67

4.3.7 Yeasts involved in *Hausa koko* fermentation

More than 70 % of the 250 yeast cultures isolated from the different production sites were budding single, double or multiple cells that were either round or oval in shape. Fifty-eight of the yeasts isolates selected and Sanger sequenced, were identified using the National Center for Biotechnology Information (NCBI) database as *Saccharomyces cf. cerevisiae/paradoxus* (41.4 %) with accession number KY109426.1, *Saccharomyces cerevisiae* (31.0 %) with accession number MK908003.1, *Pichia kudriavzevii* (13.8 %) with accession number MH545928.1, *Clavispora lusitaniae* (8.6 %) with accession number MT032430.1 and *Candida tropicalis* (5.2 %) with accession number MH545915.1. The percentage identities of the isolates to similar sequences in

the database showed between 99-100 % of similarity within each strain type and are presented in Table 6. The type and percentage occurrence of the yeast from the different production sites are shown in Table 7.

Table 6: Yeast species from *Hausa koko* identified using typed strains only

Site/Product	Isolate Code	Identification Yeast	% Identity	Accession number
Dodowa/Sediment	YDOD-Sdf	*Clavispora lusitaniae*	100	MT032430.1
Mankessim/Supernatant	YMAN-Sud	*Saccharomyces cerevisiae*	100	MK908003.1
Accra/Supernatant	YAMZ-Sug	*Pichia kudriavzevii*	100	MH545928.1
Accra/*H.koko*	YAMZ-Kb	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Tamale/*H.koko*	YTAD-Kd	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Sunyani/*H.koko*	YSUN-Kj	*Saccharomyces cerevisiae*	99	MK908003.1
Dodowa/Sediment	YDOD-Sdb	*Saccharomyces cerevisiae*	100	MK908003.1
Accra/*H.koko*	YAMZ-Kh	*Pichia kudriavzevii*	99	MH545928.1
Sunyani/Sediment	YSUN-Sda	*Saccharomyces cerevisiae*	100	MK908003.1
Accra/Grains	YAMZ-Da	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Tamale/Sediment	YDOD-Sda	*Saccharomyces cerevisiae*	100	MK908003.1
Accra/Grains	YAMZ-Db	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Tamale/Supernatant	YTAD-Suf	*Pichia kudriavzevii*	100	MH545928.1
Sunyani/Supernatant	YSUN-Sud	*Saccharomyces cerevisiae*	99	MK908003.1
Dodowa/Grains	YDOD-Dc	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Tamale/Grains	YTAD-De	*Saccharomyces cerevisiae*	99	MK908003.1
Tamale/Sediment	YTAD-Sdc	*Pichia kudriavzevii*	100	MH545928.1
Tamale/*H.koko*	YTAD-Kg	*Pichia kudriavzevii*	100	MH545928.1
Tamale/*H.koko*	YTAD-Kh	*Pichia kudriavzevii*	100	MH545928.1
Sunyani/Supernatant	YSUN-Suf	*Saccharomyces cerevisiae*	100	MK908003.1
Dodowa/Supernatant	YDOD-Sud	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Dodowa/*H.koko*	YDOD-Ke	*Candida tropicalis*	100	MH545915.1
Dodowa/Sediment	YDOD-Sdg	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Mankessim/*H.koko*	YMAN-Kg	*Pichia kudriavzevii*	100	MH545928.1
Sunyani/24 h	YSUN-24a	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1

Tamale/Grains	YTAD-Da	*Candida tropicalis*	99	MH545915.1
Dodowa/12 h	YDOD-12e	*Saccharomyces cf. cerevisiae/paradoxus*	100	KY109426.1
Dodowa/Supernatant	YDOD-Sua	*Saccharomyces cf. cerevisiae/paradoxus*	100	KY109426.1
Dodowa/12 h	YDOD-12g	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Sunyani/Sediment	YSUN-Sdb	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Sunyani/Sediment	YSUN-Sdi	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Dodowa/12 h	YDOD-12j	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Mankessim/Supernatant	YMAN-Sue	*Saccharomyces cerevisiae*	100	MK908003.1
Tamale/12 h	YTAD-12g	*Saccharomyces cerevisiae*	100	MK908003.1
Dodowa/Supernatant	YDOD-Suh	*Candida tropicalis*	100	MH545915.1
Accra/Sediment	YAMZ-Sdb	*Saccharomyces cerevisiae*	100	MK908003.1
Sunyani/*H.koko*	YSUN-Kf	*Saccharomyces cerevisiae*	99	MK908003.1
Mankessim/24 h	YMAN-24g	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Mankessim/24 h	YMAN-24j	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Mankessim/Sediment	YMAN-Sdc	*Saccharomyces cerevisiae*	100	MK908003.1
Tamale/Sediment	YTAD-Sdb	*Clavispora lusitaniae*	100	MT032430.1
Mankessim/24 h	YMAN-24d	*Saccharomyces cerevisiae*	100	MK908003.1
Sunyani/Grains	YSUN-Dc	*Clavispora lusitaniae*	100	MT032430.1
Accra/Sediment	YAMZ-Sdc	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Sunyani/24 h	YSUN-24b	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Sunyani/24 h	YSUN-24h	*Saccharomyces cf. cerevisiae/paradoxus*	100	KY109426.1
Accra/*H.koko*	YAMZ-Ka	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Accra/24 h	YAMZ-24g	*Clavispora lusitaniae*	99	MT032430.1
Mankessim/Grains	YMAN-Dd	*Clavispora lusitaniae*	99	MT032430.1
Sunyani/24 h	YSUN-24i	*Saccharomyces cerevisiae*	100	MK908003.1
Dodowa/Supernatant	YDOD-Suc	*Saccharomyces cerevisiae*	100	MK908003.1
Mankessim/*H.koko*	YMAN-Kc	*Pichia kudriavzevii*	99	MH545928.1
Accra/24 h	YAMZ-24i	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Tamale/12 h	YTAD-12j	*Pichia kudriavzevii*	100	MH545928.1
Tamale/12 h	YTAD-12a	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Tamale/Supernatant	YTAD-Sud	*Saccharomyces cf. cerevisiae/paradoxus*	99	KY109426.1
Tamale/12 h	YTAD-12f	*Saccharomyces cerevisiae*	99	MK908003.1
Tamale/12 h	YTAD-12b	*Saccharomyces cf. cerevisiae/paradoxus*	100	KY109426.1

Table 7: Type and percentage occurrence of yeast identified from the different production sites

Production Site	Yeast specie	Percentage (%) Occurrence
Dodowa	*Clavispora lusitaniae*	7.69
	Saccharomyces cerevisiae	23.08
	Saccharomyces cf. cerevisiae/paradoxus	53.85
	Candida tropicalis	15.38
Tamale	*Pichia kudriavzevii*	38.46
	Saccharomyces cerevisiae	23.08
	Candida tropicalis	7.69
	Clavispora lusitaniae	7.69
	Saccharomyces cf. cerevisiae/paradoxus	23.08
Sunyani	*Saccharomyces cerevisiae*	50
	Saccharomyces cf. cerevisiae/paradoxus	41.67
	Clavispora lusitaniae	8.33
Mankessim	*Saccharomyces cerevisiae*	44.45
	Pichia kudriavzevii	22.22
	Saccharomyces cf. cerevisiae/paradoxus	22.22
	Clavispora lusitaniae	11.11
Accra -Madina Zongo	*Pichia kudriavzevii*	20
	Saccharomyces cf. cerevisiae/paradoxus	60
	Saccharomyces cerevisiae	10
	Clavispora lusitaniae	10

The most frequently isolated yeast species from the *Hausa koko* production sites was *Saccharomyces cf. cerevisiae/paradoxus.* In addition to *Saccharomyces cf. cerevisiae/paradoxus* the 28S rRNA sequencing also identified some isolates as *Saccharomyces cerevisiae.* Both *S. paradoxus* and *S. cerevisiae* were associated with the fermentation of millet in *Hausa koko* production at all the production sites.

Pichia kudriavzevii was the third most dominant yeast (13.8 %) isolated in *Hausa koko* production. It was isolated at the Tamale, Mankessim and Accra production sites.

Clavispora lusitaniae (8.6 %) and *Candida tropicalis* (5.2 %) were the other yeast species identified and were present in low numbers. Although they were not the predominant species, *Clavispora lusitaniae* was isolated at all the production sites whilst *C. tropicalis* were isolated at Tamale and Dodowa sites. It was observed that same strains were circulating in different environments.

4.4 Discussion

4.4.1 Lactic acid fermentation of *Hausa koko*

The pH of the fermenting substrates, the steeped millet grains or the millet slurry, decreased steadily during the production of *Hausa koko*. Generally, significant differences ($p \leq 0.05$) existed in the pH reductions at the different stages of processing among the different processors. The decrease was from pH 4.35 ± 0.01 to 4.08 ± 0.01 during the steeping of the millet grains and from pH 3.65 ± 0.01 to 3.27 ± 0.01 during the fermentation of the millet slurry. The decrease in pH may be due to production of lactic acid, which increased with increasing population of the LAB. The pH of the grains ranged from 6.02 ± 0.01 - 6.53 ± 0.01 but decreased in the final product *Hausa koko* (pH 3.51 ± 0.01 - 3.99 ± 0.01) from the different processors as expected. This may be attributed to increase in the population of fermenting microbes like LAB with the production of acidic metabolites. Production of sour food products involving an increase in lactic acid population and decrease in pH is very common in Ghana and West Africa as a whole. In Ghana, this trend has been reported in different fermented foods (Annan et al., 2015; Atter et al., 2014; Halm et al., 2004; Amoa-Awua et al., 1996). According to Poutanen et al., (2009), the metabolic activities of fermenting microorganisms during cereal fermentation at temperate conditions usually produces mainly lactic and acetic acids resulting in the lowering of the pH. In Nigeria, Wakil & Daodu (2011) reported a reduction in pH from 5.7 to 3.5 during *ogi* production from maize, In Benin, Houngbédji et al., 2018 reported reductions from mean values of 5.4 at 0 h to 4.1 at 36 h of fermentation during *mawè* production. The low pH of *Hausa koko* contributes to its safety as a food product.

Several different species of lactic acid bacteria were isolated at the different stages of *Hausa koko* production and also at the different production sites. The host of lactic acid bacteria encountered at the different stages of *Hausa koko* production is likely to have originated from the raw materials and processing equipment as suggested by Jespersen (2003) in the fermentation of African indigenous foods with reference to yeast sources. There was a steady increase in the population of LAB by 4 log units during the soaking of the millet grains through to the end of the fermentation of the millet slurry which had separated into a supernatant and a sediment. The final LAB count of the supernatants were in the range of log 7.76 CFU/g to log 8.93 CFU/g and the sediments log 7.64 to log 8.94 CFU/g. Generally, the increase in population were significant ($p \leq 0.05$) at the different stages of processing among the different processors.

In the present work, the most frequently occurring LAB responsible for the fermentation of millet grains and millet slurry during *Hausa koko* production were identified (Table 3), based on the samples from the different sites. *L. pontis, L. fermentum, L. reuteri, P. pentosaceus, P. acidilactici* and *W. confusa* appear to be more consistent in the fermentation of millet during *Hausa koko* production. These results are similar with the findings of Lei & Jakobsen (2004) who had studied *Hausa koko* fermentation in the Tamale municipality based on sequencing of the 16S rRNA gene 15 years earlier, who isolated *L. fermentum, W. confusa, Pediococcus spp* and *L. salivarius.* In the present work *L. salivarius* was not isolated in *Hausa koko* fermentation, however a lot more LAB species were encountered including *L. pontis, L. reuteri, L. paracasei* and *S. harbinensis.* Again, more LAB species were identified at each processing stage than was reported (Lei & Jakobsen, 2004). A comparison of the findings of Lei & Jakobsen (2004) and the present study is given in Table 9. Two reasons may account for the additional species reported in the present work. Firstly, samples were taken from five regions which represents a wider geographical area in comparison

to the work of Lei & Jakobsen (2004) whose samples were taken from only one of the regions included in the present work. Also, the LAB isolates were identified by whole genome sequencing (WGS) in the present work which has a higher discriminatory power in distinguishing between different species compared to the sequencing of only the 16S rRNA gene and the fact that WGS method are more precise. In the present work *Limosilactobacillus pontis* was found to totally dominate the LAB population of the samples taken from Tamale though it was not reported by Lei & Jakobsen (2004) whose study was carried out in the same area but 15 years earlier. *L. pontis* was identified in three out of five production sites located in Tamale, Dodowa and Accra, although the species has not previously been reported in traditional food fermentation in Ghana.

Limosilactobacillus pontis is a heterofermentative thermophilic acid tolerant lactobacilli that has been reported to be associated with flour sourdough fermentation (De Vuyst & Neysens, 2005) and in cereal sourdough fermentation (Vogelmann et al., 2009). Abegaz (2007) also intermittently isolated *L. pontis* during the spontaneous fermentation of Ethiopian non-alcoholic cereal beverage, *borde* and from *mursik* fermented milk from Kenya (Nieminen et al., 2013).

Limosilactobacillus reuteri was isolated in four out of the five production sites. *L. reuteri* is a heterofermentative LAB which produces lactic and acetic acids, ethanol and carbon dioxide with high tolerance in low pH and bile salts (Whitehead et al., 2008; Jacobsen et al., 1999). *L. reuteri* normally resides in the gastrointestinal tract of humans and animals with the capability of producing organic acids, ethanol and enzymes. *L. reuteri* produces the enzyme reuterin which inhibits the growth of some harmful Gram negative and Gram positive bacteria, along with yeasts, moulds and protozoa. It can secret sufficient amounts of reuterin to inhibit the colonization and growth of harmful gut organisms, without killing beneficial gut bacteria, allowing *L. reuteri* to remove gut invaders while keeping normal gut flora intact, thus, benefiting the immune system of

the host (Mu et al., 2018). *L. reuteri* also produce other antimicrobial substances that competes against pathogenic microbes and adheres to the epithelial cells. Some strains also produce vitamins B9 and B12 (Mu et al., 2018; Hammes & Hertel, 2006; Walter et al., 2011). These together with its resistance properties makes them typical probiotic strains (Whitehead et al., 2008; Jacobsen et al., 1999). *L. reuteri* was one of the probiotic LAB's used to study the physicochemical composition and acceptance of fermented cereal beverages formulated with different human derived LAB strains (Salmerón et al., 2015).

Table 8: Similarities between the bacterial population of the current work and those reported by Lei & Jakobsen (2004).

Production Stages (Current Study)	Identified LAB (Current Study)	Production Stages (Lei & Jakobsen, 2004)	Identified LAB (Lei & Jakobsen, 2004)
Dry Millet Grains	*L. pontis*	Dry Millet Grains	*L. fermentum*
	L. fermentum		*W. confusa*
	P. acidilactici		*P. spp*
	P. pentosaceus		
	L. reuteri		
12 & 24 h Fermented Millet	*L. fermentum*	Milled Millet	*L. fermentum*
	W. confusa		*W. confusa*
	L. pontis		*L. salivarius*
	L. reuteri		
	P. pentosaceus		
Supernatant	*L. pontis*	Top Layer (KSW)	*L. fermentum*
	L. fermentum		*W. confusa*
	L. reuteri		*L. salivarius*
	L. paracasei		*L. paraplantarum*
	P. pentosaceus		*P. acidilactici*
	W. confusa		*P. pentosaceus*
	P. acidilactici		
Sediment	*L. fermentum*	Bottom Layer	*L. fermentum*
	L. pontis		*W. confusa*
	W. confusa		*L. salivarius*
	L. reuteri		*P. spp*
	P. acidilactici		
	S. harbinensis		
	L. paracasei		
	P. pentosaceus		
Hausa koko	*L. pontis*	*Koko*	*L. fermentum*
	P. acidilactici		*W. confusa*
	L. plantarum		
	P. pentosaceus		
	L. fermentum		
	S. harbinensis		
	L. reuteri		

NB: KSW=Koko sour water; *L.* in the current study is *Limosilactobacillus* while in Lei & Jakobsen (2004), *L.* represents *Lactobacillus*

The homofermentative *Pediococcus acidilactici* was the only one isolated from all the five production sites. They have antagonistic activities against some Gram-positive and Gram-negative organisms in conjunction with lactic and acetic acids with possible protection against diseases in the gastrointestinal tract (Ferguson et al., 2010). *P. acidilactici* is common in fermented dairy, meat and vegetable products and some strains produce pediocin which also inhibits several spoilage and pathogenic organisms. They have been used as flavour enhancers due to the formation of volatile compounds during cheese fermentation (Carafa et al., 2015; Dina et al., 2013; Ammor & Mayo, 2007; Barros et al., 2001). Adimpong et al., (2012) have reported its presence in indigenous African fermented foods, in *togwa*, a Tanzanian cereal fermented beverage by Mugula et al., (2003a), in *gowé* made from sorghum in Benin (Vieira-Dalodé et al., 2007), in the production of *doklu* from maize in Côte d'Ivoire (Assohoun-Djeni et al., 2016). Sekwati-Monang (2011) used a combination of *P. acidilactici* and *S. harbinensis* as starter culture for the fermentation of sorghum during *ting* production in Botswana. Starkutė et al., (2017) has reported a reduction in mycotoxin levels in cereal by-products by using *P. acidilactici* as starter culture for the fermentation.

Limosilactobacillus fermentum was also isolated in four out of the five production sites, hence is one of the dominant LAB in *Hausa koko* fermentation. *L. fermentum* is a heterofermentative LAB which is one of the dominant LAB in the traditional fermentation of cereals in Africa. The traditional foods in which *L. fermentum* are associated with include *doklu* (Assohoun-Djeni et al., 2016), *ogi* (Omemu & Faniran, 2011), *kunun-zaki* (Agarry et al., 2010), *nsiho* (Annan et al., 2015), *burukutu* (Atter et al., 2014), *mahewu* (Pswarayi & Gänzle, 2019), *dolo* and *pito* (Sawadogo-Lingani et al., 2007) etc. Lei & Jakobsen (2004), had reported *L. fermentum* to be predominant in *Hausa koko* fermentation.

An important starter culture bacterium involved in fermenting foods with good preservation characteristics is the homo fermentative *Pediococcus pentosaceus*. It was isolated from four of the production sites. It has antimicrobial and antioxidant properties, is able to tolerate acids and bile salts, improve safety quality, extend shelf life and affect flavour characteristics on food products (Kumar et al., 2017; Osmanağaoğlu et al., 2001). *P. pentosaceus* is a potential probiotic LAB that is able to survive in low pH and bile salt and was isolated from *omegisool* a tradition Korean fermented millet alcoholic beverage. These isolates exhibited resistance to different antibiotics, adhesion capacity and antioxidant activity (Oh & Jung, 2015). *P. pentosaceus* isolated from maize leaf was able to decrease the production of fumonisin contamination in maize kennels as well as liquid medium (Dalié et al., 2012). Their association has also been reported in the fermentation of *borde*, a cereal beverage from Ethiopia (Abegaz, 2007) and during the fermentation of a millet-based food, *dèguè*, consumed in Burkina Faso (Ouattara et al., 2015).

Weissella confusa which is heterofermentative was also isolated in three out of the five production sites and are associated with a variety of fermented foods (Fusco et al., 2015; Lee et al., 2012). Several strains of *W. confusa* have been established as probiotic in nature vastly because of their antimicrobial properties, with few strains identified as opportunistic bacteria. They showed antifungal activity against *Penicillium roqueforti*, *Aspergillus niger*, and *Endomyces fibuliger* during wheat fermentation which was characterised by low pH and high volumes of lactic and acetic acid production (Valerio et al., 2009). Nam et al., (2002) have proposed *W. confusa* as a probiotic starter culture due to its ability to inhibit *Helicobabacter pylori* which causes stomach ulcers and other inflammations. Houngbédji et al., (2018) reported the occurrence of *W. confusa* mainly at the onset of a cereal based food *mawè*, fermentation in Benin. In this study, though *W. confusa* was isolated in low numbers, its presence in three out of the five production sites indicates

its widespread occurrence in *Hausa koko* fermentation. It has been associated with other fermented pearl millet foods including *fura* and *Kimere* (Owusu-Kwarteng et al., 2012; Njeru et al., 2010).

Lacticaseibacillus paracasei was isolated at the Accra and Tamale production sites whilst *S. harbinensis (*formally *L. harbinensis)* and *L. plantarum* were isolated only at Dodowa. *L. plantarum* and *L. paracasei* subsp. *paracasei* have been reported in *bushera* in Uganda (Muyanja et al., 2003). *L pentosus, L. plantarum* and *L. paraplantarum* share similar phenotypic characteristics and similar 16S rRNA gene sequences (≥ 99 %) which makes it difficult to differentiate among them (Torriani et al., 2001). *L. plantarum* has been reported in the fermentation of maize, millet and sorghum in the production of *akamu* and *kunu-zaki* (Nwachukwu et al., 2010). The presence of *L. paraplantarum* was reported at the initial stages of millet fermentation during *fura* production in Ghana by Owusu-Kwarteng et al. (2012). Facultative heterofermentative *S. harbinensis* has been reported in sorghum sourdough fermentation (Sekwati-Monang et al., 2012), and *S. harbinensis, L. plantarum,* and *L. paracasei* in raw milk and cheese fermentation (Agostini et al., 2018).

4.4.2 Involvement of yeast in *Hausa koko* fermentation

According to De Vuyst and Neysen (2005), lactic acid bacteria and yeast occur naturally in the ecological niche of cereals playing significant roles during their fermentation. The presence of yeasts has been reported in several fermented foods and their symbiotic relationship with lactic acid bacteria in such fermentations has also been established. Assohoun-Djeni et al., (2016) reported similar populations of LAB and yeast during the fermentation of maize flour during *doklu* production where LAB and yeast increased from log 4.2 to 9 CFU/g and log 4.9 to 7.8 CFU/g respectively. The increasing trend in yeast population can be attributed to their great growth rate

compared to other microorganisms (Holzapfel, 2002). In the present study, the yeast population during *Hausa koko* production was dominated by *Saccharomyces cf. cerevisiae/ paradoxus* and *Saccharomyces cerevisiae*. They accounted for about 70 % of the total yeast population in *Hausa koko* production and were found at all five production sites located in the five different geographical regions of Ghana. *S. paradoxus* is a wild yeast and the closest known species to *S. cerevisiae* (Kowallik et al., 2015; Fay & Benavides, 2005). The genome of *S. paradoxus* is highly conserved when compared to *S. cerevisiae.* In coding regions, the genome of *S. paradoxus* shares 90 % of identity with the genome of *S. cerevisiae*, and in the intergenic regions it has 80 % homology (Kellis et al., 2003). *S. paradoxus* is almost morphologically indistinguishable from *S. cerevisae* in nearly all aspects of morphology, metabolism, and its life cycle (Sniegowski et al., 2002; Sweeney et al., 2004). Identification of the yeasts was by ITS PCR followed by Sanger 28S rRNA sequencing which probably made it possible to identify *S. paradoxus* and *S. cerevisiae* as different yeasts species.

The yeasts population in most African fermented cereal foods have been reported to be dominated by *Saccharomyces cerevisiae.* These include *mawè* (Houngbédji et al., 2018, *ogi* (Kigigha et al., 2016; Izah et al., 2016), cereal based fermented foods (Achi & Ukwuru, 2015), *burukutu* (Atter et al., 2014), palm wine (Amoa-Awua, et al., 2007) and many others (Greppi et al., 2013; Achi & Ukwuru, 2015). *Saccharomyces paradoxus* on the other hand has only been reported in a few instances; *akamu*, a cereal based complementary food (Obinna-Echem et al., 2014) and in sorghum beer from Ghana and Burkina (Naumova et al., 2003). It is noted that in the two instances that the presence of *S. paradoxus* was reported in the African traditional foods, the authors used molecular characterisation involving sequencing of the internal transcribed spacer regions (ITS1 and ITS2). It is therefore possible that in some of the instances where *S. cerevisiae* has been reported and

identification was by phenotypic characterisation based mainly on the fermentation and utilization of different sugars, the yeasts could have been *S. paradoxus*. This is because they share the same phenotypic characteristics and would be identified as *S. cerevisiae* using API kit. *S. cerevisiae* and *S. paradoxus* co-exist in similar environment and exhibit indistinctive phenotypic characteristics, identical spore phenotypes, sugar utilization and assimilation reactions (Sniegowski et al., 2002). *S. paradoxus* is the undomesticated relative of *S. cerevisiae* (Kowallik et al., 2015; Tsai et al., 2008). It is therefore likely that *S. paradoxus* which is a wild form of *S. cerevisiae* plays a greater role in the fermentation of indigenous Africa fermented foods than has been reported.

The other yeasts found in *Hausa koko* production in the present work were *Pichia kudriavzevii/Candida krusei, Clavispora lusitaniae,* and *Candida tropicalis. P. kudriavzevii* is the teleomorph of *Candida krusei* with some few strains being opportunistic pathogens (Johansen et al., 2019). The presence of *Candida krusei/ Pichia kudriavzevii* has been reported extensively in African fermented cereal and other foods including *mawe* (Greppi et al., 2013), *fura* (Pedersen et al., 2012), *gowe* (Vieira-Dalode` et al., 2007), *agbelima* (Amoa-Awua et al., 1997). *Clavispora lusitaniae* and *Candida tropicalis* have been reported in other fermented cereals in Africa. *Clavispora lusitaniae* in *obushera* (Mukisa et al., 2012), *ogi* (Greppi et al., 2013; Omemu et al., 2007) and *C. tropicalis* in *togwa* (Mugula et al., 2003a). Yeasts cause acidification, and produce ethanol, carbon dioxide, extracellular enzymes production, as well as generate of flavour compounds, bio-preservations and many others (Furukawa et al., 2013; Omemu et al., 2007; Amoa-Awua et al., 2007; Oyewole, 2001).

4.4.3 Microbial contaminants in *Hausa koko* production

According to De Vuyst and Neysens (2005) Enterobacteriaceae, *Staphylococcus aureus, Bacillus cereus* and many other organisms form part of the microflora of cereals. However, the presence of Enterobacteriaceae, *S. aureus,* and *E. coli* at different stages of *Hausa koko* was undesirable and give an indication of poor hygienic practices which are obvious in the traditional production practices of *Hausa koko*. *B. cereus* was not encountered at any of the production sites and also *E. coli* was not isolated at any of the production sites after fermentation of the millet slurry. In the ready to eat *Hausa koko* porridge, Enterobacteriaceae and *B. cereus* were found in the sample from Sunyani, and Enterobacteriaceae and *E. coli* in the sample from Mankessim. Generally, the counts of these pathogenic and indicator organisms which were present at the start of processing reduced steadily during production (Figure 13). This was due to production of lactic acid during fermentation and competitive exclusion due to over population of lactic cultures, thus lowering pH, and possibly production of other antimicrobial compounds also during the fermentation as most of the LAB were heterofermenters.

Microbial counts obtained from the different processors, interaction and critical observation of the entire process during the sampling depicts some form of contamination along the production processes. The sources of contamination of *Hausa koko* during production could be from the millet grains, which often contain small stones/sand and other foreign materials. The presence of the stones is due to threshing of the harvested millet on the bare ground by farmers. The grains may also be stored under unhygienic conditions and poorly handled during transportation. Cleaning of millet grains during processing is very tedious and time consuming as the processor must carefully pick out or sieve out the stones during washing of the grains. This rather tedious time-consuming manual operation requires large volumes of water as well, and processors may be tempted to

economize on the use of water, hence the millet grains may not be washed thoroughly. After steeping also, some processors do not wash the steeped grains adequately. Laca et al., (2006) have explained that the microbial population on wheat grains differs between varieties and majority of bacteria on the grains are strongly attached to the grains such that they cannot be easily removed by simple agitation in a liquid but their removal is very important for food safety. Similar to our results, bacteria and fungi contamination of grains have been reported (Oranusi et al., 2017). Microorganisms such as *Staphylococcus aureus, Escherichia coli, Klebsiella aerogenes, Proteus vulgaris*, *Bacillus spp* and many others were reported in unmalted pearl millet grains as well as at different malting stages (Badau, 2006).

It was also observed that some processors do not wash at all or do not thoroughly wash the dried spices that they mill together with the steeped millet grains before it is fermented in the form of a slurry. The dry spices if not thoroughly washed, preferably with a sanitizing agent, carry their own microflora which will contaminate slurry to be fermented. Although spices are mostly added to foods to improve on the aroma, flavour and taste, they have been reported to contain pathogenic bacteria and fungi, which may cause foodborne illnesses (Bakobie et al., 2017; Ahene et al., 2011). Other possible sources of *Hausa koko* microbial contamination may include use of inadequately cleaned milling machine, which will harbor microorganisms from the previous materials milled. Accumulation of grain flour in the milling machine for long period is a reservoir for microbial contaminants which can cause cross contamination (Sabillón et al., 2021). Microbial contaminants may also be introduced into the process when the freshly prepared slurry is strained using a muslin or cheese cloth contaminated with the pathogenic microorganisms from previous use. Processors may use a muslin or cheese cloth a few times without washing it thoroughly. A processor may also contaminate the materials with their bare hands and other contact surfaces if they are not observing

strict personal hygiene since they do not wear gloves (Kusumaningrum et al., 2003; Hilton & Austin, 2000; Scott & Bloomfield, 1990).

In the present work on *Hausa koko* production, fortunately the population of the pathogenic organisms reduced very drastically or disappeared completely near the end of the process. This was attributable to the very low pH attained due to the production of mainly lactic acid ensuring safety of the product. Also, application of heat during the cooking of the porridge reduced the microbial loads significantly ($p \leq 0.05$) further. Enterobacteriaecie for instance ranged from log 2.49 ± 0.04 to log 6.72 ± 0.04 CFU/g in the dry millet grains but in cooked ready to eat *Hausa koko* samples, they were either not detected or present at not more than log 2.41 ± 0.13 CFU/g.

4.5 Conclusion

The central operation in the processing of millet into *Hausa koko* is fermentation, which involves the steeping of millet grains and spontaneous fermentation of the steeped grains that has been milled together with spices and made into a slurry. Fermentation in *Hausa koko* production has been confirmed to be an acidification process which involves growth of lactic acid bacteria and yeasts, resulting in the lowering of pH. The species of lactic acid bacteria responsible for the souring fermentation identified by whole genome sequencing are *Limosilactobacillus pontis*, *Limosilactobacillus fermentum*, *Pediococcus acidilactici*, *Pediococcus pentosaceus* and *Limosilactobacillus reuteri*. Others which occur in very small numbers are *Weissella confusa*, *Schleiferilactobacillus harbinensis*, *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei*. The pH reduced from a range of 6.02 ± 0.01 to 6.53 ± 0.01 in the grains to 3.51 ± 0.01 to 3.99 ± 0.01 in *Hausa koko*. The yeasts species which grew along the lactic acid bacteria were

identified by Sanger sequencing to be *Saccharomyces cf. cerevisiae/paradoxus*, *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *Clavispora lusitaniae* and *Candida tropicalis*. The presence of some indicator and pathogenic organisms were found during *Hausa koko* production though their numbers reduced drastically through the process to the final product (the ready-to-eat porridge). These organisms were Enerobacteriaecie, *Staphylococcus aureus*, *Bacillus cereus* and *E. coli* and shows that there is the need to improve hygienic practices at the production sites to improve the safety of *Hausa koko*.

CHAPTER FIVE

5.0 Technological and probiotic properties of LAB and yeast from *Hausa koko*, a millet base porridge

5.1 Introduction

Fermented foods possess antimicrobial and therapeutic functions provided by the microorganism they contain (Mokoena et al., 2016; Jespersen 2003). They therefore serve as a good source for screening and identifying novel microorganisms with exceptional technological and/or probiotic properties and have high performance in the fermentation environment (Sim et al., 2012).

The use of such fermenting organisms with technological and probiotic features is one of the means to decrease or inhibit growth of pathogens and food spoilage organisms during fermentation and improve the nutritional and organoleptic quality of the fermented food (Bourdichon et al., 2012). Extensive research has been conducted on the technological properties and probiotic characteristics of several microorganisms from spontaneously fermented foods, mainly lactic acid bacteria (LAB) and yeast. These technological properties are characterised by evaluating the implicit characteristics of LAB such as rapid acidification of fermenting substrates, production of antimicrobials such as organic acids, small chain fatty acids, bacteriocin activity, exopolysaccharide production, antagonistic characteristics against microbial pathogens, enzyme activity, production of volatile compounds, production of other inhibitory and beneficial compounds (De Souza Motta & Gomes, 2015; Owusu-Kwarteng et al., 2015). Probiotics are mainly made from species from the gastro intestinal tract (GIT) and regarded as functional ingredients which beside the basic nutrients they provide also promotes good health benefits to the

host in several ways (Enujiugha & Badejo, 2017; Nagpal et al., 2007). Probiotic properties are also characterised by evaluating their implicit characteristics such as survival at extreme conditions like low-neutral pH, low oxygen level, bile, enzymes, temperature and so on in fermented foods and the human gut (Enujiugha & Badejo, 2017).

Sixteen (16) *Lactobacillus* (now *Limosilactobacillus*) *fermentum* strains from West African fermented millet were reported to have technological and probiotic characteristics (Owusu-Kwarteng et al., 2015). Similar probiotic yeast strains with high hydrophobicity, auto-aggregation, cholesterol removal ability, phytase, lipase and protease activities were also reported in yeast strains from fermented cereal based foods from Nigeria (Ogunremi et al., 2015).

The use of LAB with good technological properties and as probiotics is essential for the reason that lactic acid fermentation imparts several positive effects on the final product compared to the unfermented substrate as well as health benefits to the consumer (Enujiugha & Badejo, 2017). The types and amount of metabolites produced during the fermentation depends on the population, types and characteristics of the probiotic LAB strains available and thus results in different aromas, textures and tastes of fermented foods (Božanić et al., 2003; Messens & De Vuyst, 2002). Several authors have established the production of lactic and acetic acids as the main antimicrobial substance by LAB during fermentation of cereals (Madigan et al., 2012; Theron & Lues, 2010; 2007; Min et al., 2007; Nes & Johnsborg, 2004). These organic acids impart characteristic sourness to fermented foods and play an important role in controlling some pathogens and spoilage organisms present in spontaneously fermented foods. Other antimicrobials produced include hydrogen peroxide (H_2O_2) which can accrue to levels that inhibit target spoilage and pathogenic organisms (Enitan et al., 2011; Adesokan et al., 2010; Zalán et al., 2005; Ito et al., 2003). LAB

also produce exopolysaccharides (EPS) that contribute to characteristic rheology and textures of fermented foods (Oleksy & Klewicka, 2018; Sanalibaba & Çakmak, 2016; Górska et al., 2007; Ruas-Madiedo et al., 2002; De Vuyst et al., 2001) and diacetyl (Ammor et al., 2006; Bartowsky & Henschke, 2004). Other technological properties of microbial fermenters include ability to produce a variety of enzymes such as amylases, proteases, phytases, cellulases and lipases that degrade complex substrates such as carbohydrates, proteins, lipids and cell wall components and thus improve starch and protein digestibility during cereal fermentation (Iyer & Ananthanarayan, 2008; Karovičová & Kohajdova, 2007). Some potential probiotic LAB also produces bacteriocins, the peptides or peptide complexes that have antagonistic activity against closely related bacteria strains either of the same species or across genera (López-Cuellar et al., 2016; Perez et al., 2014; De Vuyst & Leroy, 2007; Bowdish et al., 2005; Cotter et al., 2005; Jeevaratnam et al., 2005; Cleveland et al., 2001).

The potential application of yeast as probiotics has also been reported (Nayak, 2011). Just like LAB, beneficial yeast isolates involved in fermentations also possess antagonistic properties against pathogenic and food spoilage organisms. Some strains are able to withstand bile and acidic conditions as well as grow under different temperatures and NaCl conditions. These properties make them candidates for different applications in the food industry (AbdElatif et al., 2016). Their antimicrobial properties contribute to the safety and shelf life of fermented foods (Psani & Kotzekidou, 2006). Most strains of LAB are not tolerant to antibiotics, but Syal & Vohra (2013) reported the tolerance of several yeast species from fermented foods to different antibiotics. They also produce metabolites that have antioxidant properties (Abbas, 2006).

Due to the significant roles LAB and yeast play during lactic acid fermentation, they are accepted and applied as probiotic microorganisms when found in compliance with the specific guidelines

for evaluation of probiotics for food use (Morelli & Capurso, 2012). The various fermentation stages of *Hausa koko* production have been identified as a rich source of homo and hetero fermentative LAB and yeast. These isolates may serve as a good source for screening and identifying novel isolates with exceptional technological and probiotic properties. Since indigenous fermented foods like *Hausa koko* continue to be a chief source of lactic acid bacteria and yeast, more research needs to be conducted into their properties and the possible beneficial traits they may confer on consumers. In lieu of this, the technological and probiotic producing properties of LAB and yeast isolates from *Hausa koko* can be explored *in vitro* for their usage in food production to improve the quality and safety of the food. The objective of this study was therefore to evaluate the technological and probiotic properties of LAB and yeast isolates from *Hausa koko* to identify potential isolates with probiotic properties for use as starter culture or inoculum enrichment during millet fermentation in future studies.

5.2 Materials and Methods

5.2.1 Lactic acid bacteria and yeast isolates

Isolates from various *Hausa koko* production stages were used for this study. They were obtained from the following stages: dry millet (D), 12 hour fermented millet (12 h), 24 hour fermented millet (24 h), milled millet with spices (M), supernatant of slurry (Su), sediment of slurry (Sd) and *Hausa koko* (K). They were taken from sites located in Northern Region, Tamale Dabokpa (TAD); Bono Region, Sunyani (SUN); Central Region, Mankesim (MAN); Eastern Region, Dodowa (DOD) and the Greater Accra Region, Accra Madina Zongo (AMZ). All the 90 successfully sequenced LAB isolates were selected for pre-screening. These were *Limosilactobacillus pontis* (28 isolates), *Pediococcus acidilactici* (15), *Limosilactobacillus fermentum* (15), *Pediococcus pentosaceus* (10), *Limosilactobacillus reuteri* (9), *Weissella confusa* (6), *Schleiferilactobacillus harbinensis* (3), *Lactiplantibacillus plantarum (2), Lacticaseibacillus paracasei* (2). Yeast isolates, *Saccharomyces cf. cerevisiae/paradoxus* (24 isolates), *Saccharomyces cerevisiae* (17 isolates), *Pichia kudriavzevii* (9 isolates) and *Candida tropicalis* (3 isolates) were selected and screened. *Clavispora lusitaniae* (5 isolates) a known non-beneficial yeast were not selected for screening.

5.2.2 Pre-screening of LAB for technological properties

5.2.2.1 Bacteriocin gene screening

Bacteriocin detection was carried out using bacteriocine genome mining tool BAGEL4 via http://bagel.molgenrug.nl/. The BAGEL4 database can be used to identify different types of bacteriocins, including canocin, enterolysin, thermophilin, bovicin, enterocin and mutacin among others. Each genome sequence was first uploaded in FASTA format to the website and after the analyses, results were converted into a spreadsheet format using MS Excel 2016.

5.2.2.2 Selected genomic features screened

Genome mining tool PATRIC (http://patricbrc.org) database v3.6.2 was used to detect selected genomic features, including antimicrobial resistance (AMR) genes, genes related to nutritive and anti-nutritive compounds production in the isolates. The genome sequence file of each isolate was first annotated using PATRIC's Genome Annotation Service which employs the RAST (Rapid Annotation using Subsystem Technology) toolkit. With the exception of AMR, the selected genomic features were investigated in each isolate using their respective EC (Enzyme Commission) numbers after annotations were completed. This was carried out via the 'Features' option under the 'Genome view' browser. The other genomic features investigated apart from AMR genes were genes predicted to produce folate (EC No. 6.3.2.12), niacin (EC No. 3.5.1.19), thiamine (EC No. 2.7.6.2), amylase (EC No. 3.2.1.1), lysine dehydrogenase (EC No. 1.4.4.15), tyrosine 2,3 – amino mutase (EC No. 5.4.3.6), tryptophan (EC No. 4.2.1.20) and riboflavin (EC No. 2.5.1.9).

5.2.3 Technological and probiotic properties of lactic acid bacteria isolated from *Hausa koko* fermentation

5.2.3.1 Production of exopolysaccharides (EPS) by lactic acid bacteria

Exopolysaccharises production by lactic acid bacteria isolates was determined by the method described by Guiraud, (1998). Pure isolates cultured on MRS agar were streaked on LTV agar composed of 0.5 % (w/v) tryptone (Difco, France), 1 % (w/v) meat extract (Difco, France), 0.65 % (w/v) NaCl (Honeywell Fluka, Germany), 0.8 % (w/v) potassium nitrate (BDH, England), 0.8 % (w/v) sucrose (Fisher Scientific, UK), 0.1 % (v/v) Tween 80 (Sigma- Aldrich, USA), 1.7 %

(w/v) agar (Oxoid, England) and pH adjusted to 7.1. Plates were incubated at 30 ºC for 48 h and the colonies were examined for slime production using an inoculation loop (Knoshaug et al., 2000). A slime length of above 1.5 mm was considered positive and confirmed on MRS-Sucrose Broth prepared without glucose and peptone containing 1 % (w/v) meat extract, 0.5 % (w/v) yeast extract (Difco, France), 5 % (w/v) sucrose, 0.2 % (w/v) $K_2HPO_4.3H_2O$, 0.5 % (w/v) (BDH, England), sodium acetate trihydrate (Sigma-Aldrich, Germany), 0.2 % (w/v) triammonium citrate anhydrous (BDH, England), 0.02 % (w/v) $MgSO_4.7H_2O$, 0.005 % (w/v) manganese (II) sulphate monohydrate (BDH, England), 0.1 % (v/v) Tween 80 (Sigma-Aldrich, USA) with pH adjusted to 5.0 ± 0.2 (Pidoux et al., 1990). The isolates were cultured in 5 ml MRS-Sucrose broth, incubated at 30 ºC for 24 h, after which, 1.5 ml was pipetted out and centrifuged at 4000 x *g* for 10 min (4 ºC). 1 ml of the supernatant was pipetted into a glass tube and 1 ml of 95 % ethanol added. The formation of an opaque link at the interface was interpreted as presence of EPS. Positive isolates were noted according to the intensity of the opaque link.

5.2.3.2 Amylase production by LAB

Pure LAB isolates were cultured on MRS agar and streaked on Nutrient agar (Merck VM737743618, Germany) containing 2 % soluble starch (S9765, Sigma-Aldrich, Germany), pH 7.2, incubated anaerobically at 30 ºC in a jar for 3 days and flooded with iodine solution (1 %). The formation of a clear zone around the colonies with the rest of the plate staining blue-black was an indication of amylase production (Almeida et al., 2007).

5.2.3.3 Protease secretion by LAB

Pure LAB isolates were cultured on MRS agar and streaked on Plate Count Agar (Oxiod CM325; Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with 0.5 % casein (C8654, Sigma-Aldrich, New Zealand). Plates were incubated at 30 ºC for 3 days and flooded with 1M HCl (7647,

Sigma-Aldrich). A clear zone around the colonies indicated protease activity (Almeida et al., 2007).

5.2.3.4 Antimicrobial activity of LAB

Antimicrobial overlay assay method was used to screen for bacteriocin production. On the first day, LAB cultures were grown overnight by inoculating 100 µl of glycerol stocks in 20 mls MRS broth, incubated at 37 ºC overnight. Indicator strains (pathogenic) obtained from the Quadram Institute Bioscience Culture bank and CSIR-Food Research Institute Culture bank were used. They were cultured as follows:

Salmonella enterica sv *typhimurium* Lt2, and *Bacillus cereus* VLAG 699 were incubated in Nutrient broth (105443, Merck) at 37 ºC with agitation at 180 rpm. *Enterococcus faecium* ATCC 6057 was incubated in Brain Heart Infusion Broth, BHI (Oxiod CM1135; Oxoid Ltd., Basingstoke, Hampshire, UK) at 37 ºC with agitation at 180 rpm. *Staphylococcus aureus* FI10739 was incubated in BHI at 37 ºC with agitation at 180 rpm. *Micrococcus luteus* FI10640 was incubated in MRS Broth at 37 ºC in a static incubator *Enterococcus faecalis* FI9187 and *E. coli* RMEC0157 NCCBI 100282 were incubated in MRS Broth at 37 ºC in a static incubator.

On the second day, MRS agar containing 2 g/L $NaHCO_3$ (S/4200/60, Fisher Scientific, UK) was prepared and autoclaved at 121 ºC for 15 min, plates were poured and allowed to set. A loopsize smear of the overnight LAB culture from day 1 was made on triplicate MRS agar plates containing 2 g/L $NaHCO_3$ for each indicator strain and incubated at 37 ºC overnight. The overnight culture of the indicator strains (from day 1) were sub-cultured (100 µl) into their respective fresh broths and incubated at 37 ºC in their respective incubators. On the third day, the indicator strains were sub-cultured again into fresh broths and incubated for only 5 h. The growing LAB cultures on MRS plates were removed from the incubator and killed by exposing them to Ultra violet (UV)

light for 30 min in the trans-illuminator with the agar surface facing the UV light. Tubes of 5 ml fresh soft (0.7 %) Nutrient agar (Oxiod CM3; Oxoid Ltd., Basingstoke, Hampshire, UK) was prepared, autoclaved at 121 ºC for 15 min and kept at 55 ºC for use. For each indicator strain, 100 µl was added to a 5 ml fresh soft Nutrient agar (55 ºC), swirled quickly and overlaid on the respective plates, allowed to set and incubated overnight. They were then visually observed for clear/inhibition zones. A clear zone of more than 1 mm around a spot/smear was accepted as positive antimicrobial activity.

5.2.3.5 Bile salt tolerance by LAB

Pure LAB isolates were cultured overnight in MRS broth at 37 ºC. MRS agar containing 0.3, 0.5 and 1 % (w/v) bile salt (Taurocholic acid, Sigma-Aldrich, New Zealand) was prepared, allowed to cool, and plates poured. Ten microliters (10 µL) of the overnight bacterial culture was dropped at three different spots on the gridded agar surface and incubated at 37 ºC for 24 – 48 h. Plates were then checked for growth visually (Bancalari et al., 2020).

5.2.3.6 Low pH tolerance by LAB

The tolerance of LAB isolates was investigated by the method described by Bancalari et al., (2020) with slight modification. MRS agar adjusted to pH 3.5, 4.5 and 6.0 were prepared, cooled, poured, and allowed to set. MRS broth was used instead for pH 1.5 and 2.5 as they couldn't set. Ten microliters (10 µL) of overnight bacterial culture prepared from glycerol stock was dropped at two different spots on the gridded agar surface/broth and incubated at 37 ºC for 24 – 48 h. Plates and tubes were then checked for growth at the different pH visually.

5.2.3.7 Rate of acidification of millet slurry by LAB

Two hundred milliliters (200 ml) of 10 % (w/w volume) sterile millet broth (irradiated at 5 kGy radiation dose at the Ghana Atomic Energy Commission according to Mustapha et al., (2014) were prepared in sterile containers in duplicates. These were prepared by weighing 20 g of irradiated millet flour and adding 180 g of sterile distilled water to form the 10 % millet broth. They were inoculated with LAB cultures that were previously incubated for 16 h at 37 ºC in MRS broth to obtain a concentration of about 10^6 CFU/ml (confirmed by plating out on MRS agar). The mixtures were shaken immediately to homoginize and left at room temperature (28-30 ºC) to ferment for 12 h. A non-inoculated sterile broth was used as a negative control for comparative purposes. 10 ml each of the fementing samples were taken at 4 h intervals for determination of pH and titratable acidity. Change in pH and titratable acidity was done using Excel spreadsheet.

5.2.4 Technological and probiotic properties of yeasts isolated from *Hausa koko* fermentation

5.2.4.1 Growth of yeasts at different temperatures

The ability of yeasts to grow at different temperatures was investigated using Yeast Extract Peptone Dextose Broth (YPD) containing Bacteriological Peptone (20 g); Yeast Extract (10 g); 50 ml of 40 % (w/v) Glucose; Distilled water (950 ml), pH 6.5. The YPD broth was distributed in 5 ml tubes and sterilized at 121 ºC for 15 min. Each tube was inoculated with 50 µL (1 % v/v) of pure yeast culture and incubated at 25 ºC, 37 ºC or 42 ºC for 3-5 d. This was done in duplicates. Growth at the different temperatures was determined visually in comparison to the control samplewhich was not inoculated (AbdElatif et al., 2016; Psomas et al., 2001).

5.2.4.2 Bile tolerance of yeast isolates

Bile tolerance by yeast isolates was determined using the method described by Gotcheva et al., (2002) with slight modifications. Duplicates of 5 ml YPD containing 0.3, 0.5 or 1 % (w/v) bile salt (Taurocholic acid) were inoculated with 50 µL (1 % v/v) of pure yeast culture and incubated at 25 ºC for 3-5 d. Growth in the tubes were determined by visual examination using an uninoculated YPD broth for comparison.

5.2.4.3 Growth of yeasts at low pH

Five milliliters (5 ml) of YPD in duplicate tubes with pH adjusted to 1.5, 2.0, 3.0 or 5.5 with 1M HCL were inoculated with 50 µL (1 % v/v) of pure yeast cultures and incubated at 25 ºC for 3-5 d. Growth was determined by visual inspection in comparison to control samples (AbdElatif et al., 2016; Psomas et al., 2001).

5.2.4.4 Salt tolerance of yeasts

Yeast tolerance to different concentrations of NaCl was detected by inoculating 50 µL (1 % v/v) of pure yeast culture into YPD tubes (5 ml) containing 10 % and 20 % NaCl in duplicates, incubated at 25 ºC for 3-5 days. Growth was determined by visual inspection in comparison to the control samples (AbdElatif et al., 2016; Psomas et al., 2001).

5.3 Results

5.3.1 Selection of LAB for potential starter culture development

From the 90 LAB isolates that were sequenced and pre-screened using the genome mining tool BAGEL and PATRIC software, a total of twenty-seven (27) bacteriocin producing isolates were selected for further screening. The LAB isolates were *Limosilactobacillus pontis* (16 isolates), *Limosilactobacillus reuteri* (5 isolates), *Limosilactobacillus fermentum* (4 isolates), *Pediococcus acidilactici* (1 isolate) and *Pediococcus pentosaceus* (1 isolate). These isolates had genes predicted to produce mainly enterolysin A. All the *L. pontis, L. fermentum* and *L. reuteri* isolates showed a putative enterolysin A structural protein. The CDOD-Sdd (*P. pentosaceus*) genome showed a putative Bovicin 255, Penocin A and immunity structural protein whilst LAMZ-24f (*L. pontis*) showed Enterolysin A and Penocin A variant structural proteins. The LAMZ-De (*P. acidilactici*) genome showed both Enterolysin A and Mersacidine structural protein. The predicted gene information for these isolates are presented in Table 9 and the images for Enterolysin A, Mersacidine, Bovicin 255 and Penocin A genes are presented in Figures 16a, b, c, and d. Absence of protease, the proteolytic enzyme that breaks down protein were also predicted for these isolates.

Further pre-screening for predictive amylase producing genes, antimicrobial resistance genes and genes for other beneficial traits such as folate, niacin, thiamine, and riboflavin using their Enzyme Commission **(**EC) numbers with PATRIC software, as presented in Table 10. These isolates were therefore selected based on their predictive bacteriocin, nutrient and enzyme producing potential and tested individually for their technological properties for usage in the development of a starter culture.

Table 9: Twenty-seven (27) selected isolates having predicted bacteriocin genes and gene information from pre-screening of 90 whole-genome sequenced isolates

	Predicted Gene information						
Isolate	**ORF**	**Start**	**End**	**Strand**	**Length (bp)**	**Function (Gene code)**	**Protease gene**
LTAD-De (*L. pontis*)	orf00011	5951	8290	+	2337	64.3; Enterolysin_A (enlA)	Absent
LTAD-Dh (*L. pontis*)	orf00004	377	2761	+	2382	64.3; Enterolysin_A (enlA)	Absent
LTAD-Suc (*L. pontis*)	orf00020	7943	10480	+	2535	64.3; Enterolysin_A (enlA)	Absent
LTAD-Sue (*L. pontis*)	orf00020	7943	10480	+	2535	64.3; Enterolysin_A (enlA)	Absent
LTAD-Suf (*L. pontis*)	orf00023 orf00006	8857 6395	11043 8734	+ -	2184 2337	63.3; Enterolysin_A (enlA) 64.3; Enterolysin_A (enlA)	Absent
LTAD-Kh (*L. pontis*)	orf00012	3214	5598	-	2535	64.3; Enterolysin_A (enlA)	Absent
LTAD-Sda (*L. fermentum*)	orf00018	9049	11010	+	1959	63.3; Enterolysin_A (enlA)	Absent
LTAD-Kg (*L. pontis*)	orf00011	5695	3158	-	2535	64.3; Enterolysin_A (enlA)	Absent
LTAD-Sdh (*L. pontis*)	orf00004	377	2761	+	2382	64.3; Enterolysin_A (enlA)	Absent
LTAD-Kc (*L. pontis*)	orf00006 orf00023	6395 8857	8734 11043	- +	2337 2184	64.3; Enterolysin_A (enlA) 63.3; Enterolysin_A (enlA)	Absent
LSUN-Sdc (*L. reuteri)*	orf00018	7808	10489	+	2679	64.3; Enterolysin_A (enlA)	Absent
LMAN-Sdb (*L. fermentum*	orf00012	9433	11514	-	2079	63.3; Enterolysin_A (enlA)	Absent
LAMZ-24a (*L. pontis)*	orf00011 orf00023	6010 8856	8349 11042	+ +	2337 2184	64.3; Enterolysin_A(enlA) 63.3; Enterolysin_A (enlA)	Absent
LAMZ-24b (*L. pontis)*	orf00011 orf00032	5950 9428	8289 11614	+ -	2337 2184	64.3; Enterolysin_A (enlA) 63.3; Enterolysin_A (enlA)	Absent
LAMZ-De (*P. acidilactici)*	orf00023 orf00027	6671 8847	8074 10826	+ +	1401 1977	Mersacidin (mer) 64.3; Enterolysin_A (enlA)	Absent
LTAD-Dg (*L. pontis)*	orf00004	377	2761	+	2382	64.3; Enterolysin_A (enlA)	Absent
LAMZ-Sdh (*L. fermentum*)	orf00018	9049	11010	+	1959	63.3; Enterolysin_A (enlA)	Absent

LAMZ-24f (*L. pontis*)	orf00006 orf00013 orf00022 sORF_10 sORF_11	6395 8187 8831 10000 10072	8734 5571 11041 10182 10182	- - + + +	2337 2382 2208 183 111	64.3; Enterolysin_A (enlA) 64.3; Enterolysin_A (enlA) 63.3; Enterolysin_A (enlA) bacteriocinII; Bacteriocin_II; L_biotic_typeA; Antimicrobial17; Bacteriocin_IIc; 163.2; Penocin_A (penA) bacteriocinII; Bacteriocin_II; 163.2; Penocin_A (penA)	Absent
CDOD-Sdd (*P. pentosaceus*)	orf00009 sORF_6 sORF_7	1807 10000 10072	1980 10182 10182	- + +	171 183 111	43.2; Bovicin_255_variant(na) bacteriocinII; Bacteriocin_II; L_biotic_typeA; Antimicrobial17; Bacteriocin_IIc; 163.2; Penocin_A (penA) bacteriocinII; Bacteriocin_II; 163.2; Penocin_A (penA)	Absent
LMAN-Di (*L. reuteri*)	orf00007	8585	11329	-	2742	64.3; Enterolysin_A (enlA)	Absent
LAMZ-Sdi (*L. pontis*)	orf00029	3205	5604	-	2397	64.3; Enterolysin_A (enlA)	Absent
LTAD-12g (*L. pontis*)	orf00012	3213	5750	-	2535	64.3; Enterolysin_A (enlA)	Absent
LSUN-24g (*L. reuteri*)	orf00017	7745	10489	+	2742	64.3; Enterolysin_A (enlA)	Absent
LMAN-24c (*L. reuteri*)	orf00017	7745	10489	+	2742	64.3; Enterolysin_A (enlA)	Absent
LDOD-12b (*L. fermentum*)	orf00018 orf00002	9049 256	11010 3000	+ -	1959 2742	63.3; Enterolysin_A (enlA) 64.3; Enterolysin_A (enlA)	Absent
LDOD-Sud (*L. reuteri*)	orf00002	256	3000	-	2742	64.3; Enterolysin_A (enlA)	Absent
LTAD-12e (*L. pontis*)	orf00020	7943	10480	+	2535	64.3; Enterolysin_A (enlA)	Absent

NB: ORF = open reading frame, sORF = short open reading frame, bp= base, + = gene was found on the 5' to the 3' strand, - = gene was found on the 3' to the 5' strand

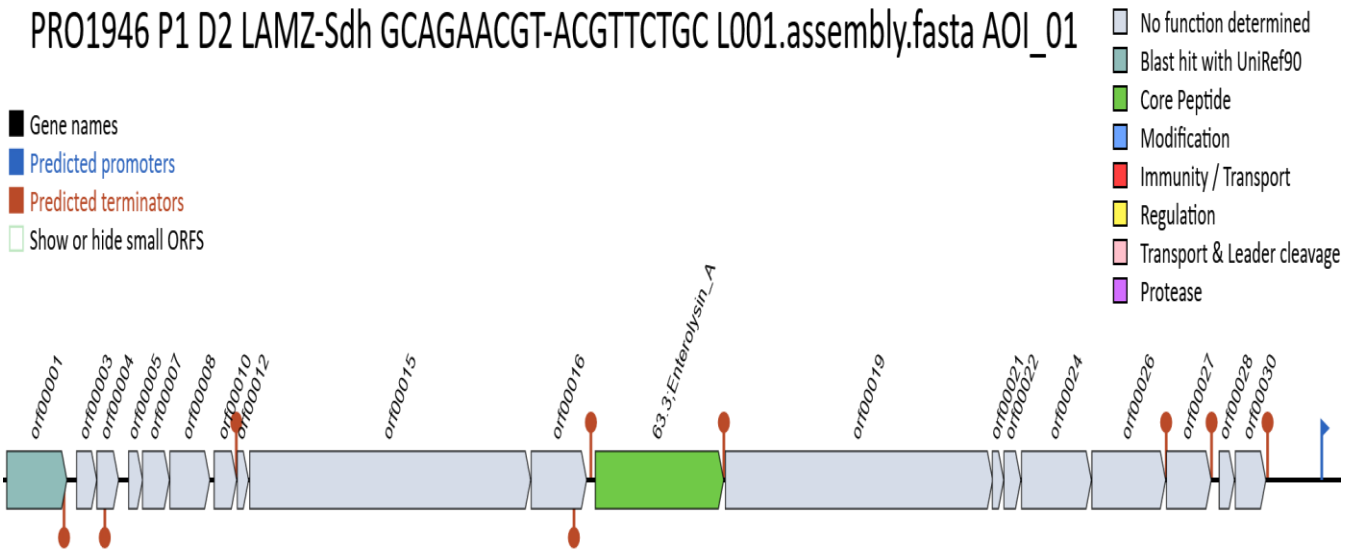


Figure 16a: Image of enterolysin A gene

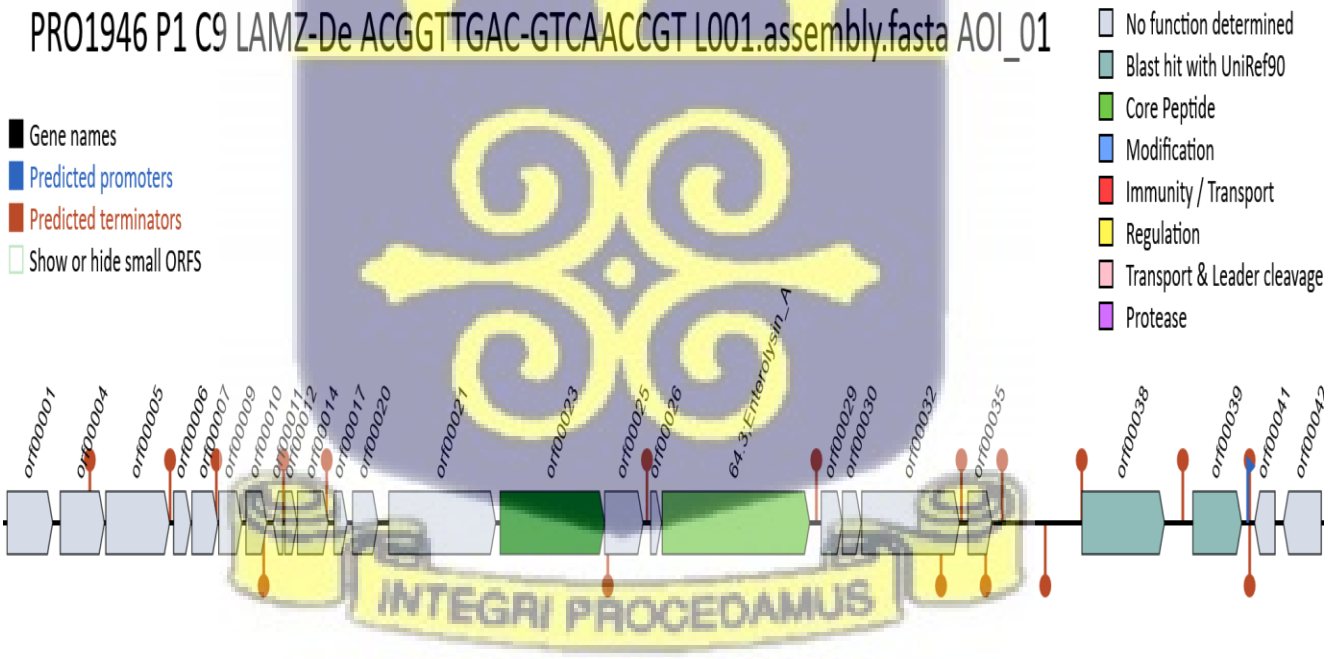


Figure 16b: Image of mersacidin (orf00023) and enterolysin A gene

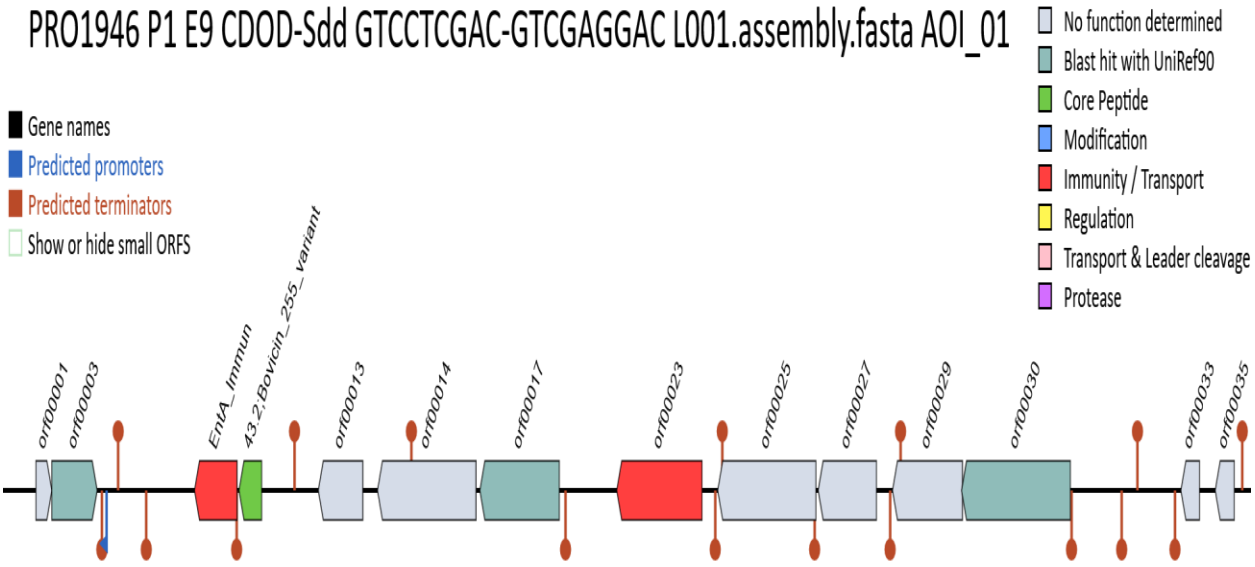


Figure 16c: Image of bovicin 255 gene

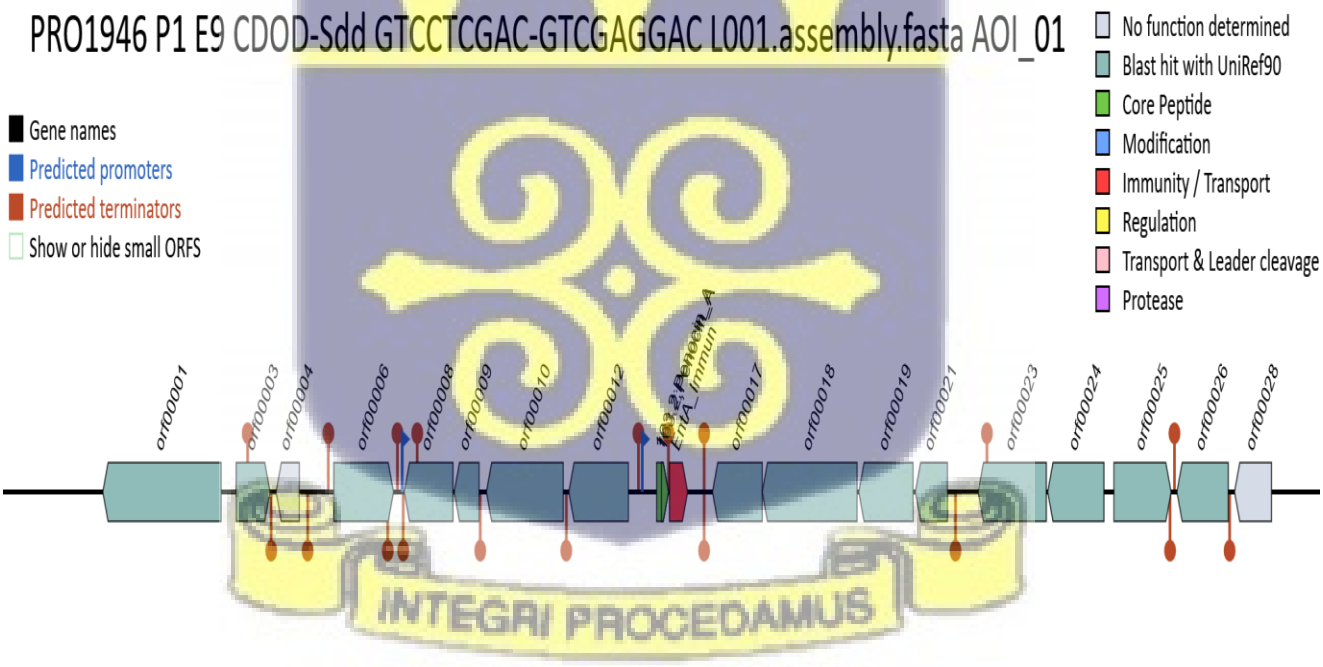


Figure 16d: Image of penocin A gene

Table 10: Twenty-seven (27) selected isolates having predicted antimicrobial resistant, nutritive and enzymatic gene information from pre-screening of 90 whole genome sequenced isolates

Isolate	Predictive AMR, nutritive and enzymatic genes								
	AMR	Tyr (5.4.3.6)	Trp (4.2.1.20)	Rf (2.5.1.9)	Fo (6.3.2.12)	Ni (3.5.1.19)	Th (2.7.6.2)	Amy (3.2.1.1)	Lyd (1.4.4.15)
LTAD-De (*L. pontis*)	0	15	0	3	3	1	21	76	4
LTAD-Dh (*L. pontis*)	0	36	0	5	5	1	23	63	11
LTAD-Suc (*L. pontis*)	0	33	0	9	5	1	23	64	6
LTAD-Sue (*L. pontis*)	0	32	0	5	9	1	101	64	6
LTAD-Suf (*L. pontis*)	0	15	0	3	3	1	57	60	4
LTAD-Kh (*L. pontis*)	0	17	0	5	5	1	23	64	6
LTAD-Sda (*L. fermentum*)	0	17	0	6	6	4	25	36	7
LTAD-Kg (*L. pontis*)	0	17	0	5	5	1	23	64	6
LTAD-Sdh (*L. pontis*)	0	17	0	5	5	1	23	63	6
LTAD-Kc (*L. pontis*)	0	15	0	3	25	1	21	60	4
LSUN-Sdc (*L. reuteri*)	0	17	1	5	5	1	24	70	5
LMAN-Sdb (*L. fermentum*)	0	16	1	5	5	3	26	78	5
LAMZ-24a (*L. pontis*)	0	15	0	3	3	1	21	60	82
LAMZ-24b (*L. pontis*)	0	15	0	3	3	1	21	60	4
LAMZ-De (*P. acidilactici*)	0	12	2	4	5	1	26	76	5
LTAD-12e (*L. pontis*)	0	17	0	5	27	1	23	64	6
LTAD-Dg (*L. pontis*)	0	17	0	5	5	1	23	63	6
LAMZ-Sdh (*L. fermentum*)	0	16	0	5	5	4	24	74	6
LAMZ-24f (*L. pontis*)	0	59	3	23	20	4	110	306	23
CDOD-Sdd (*P. pentosaceus*)	0	15	0	5	3	1	28	66	4
LMAN-Di (*L. reuteri*)	0	19	1	7	7	1	26	71	7
LAMZ-Sdi (*L. pontis*)	0	17	0	5	5	56	23	65	6
LTAD-12g (*L. pontis*)	0	19	55	7	29	1	25	64	8
LSUN-24g (*L. reuteri*)	0	17	1	5	5	1	24	70	5
LMAN-24c (*L. reuteri*)	0	17	1	5	5	1	25	70	5
LDOD-12b (*L. fermentum*)	0	17	0	6	37	4	25	73	7
LDOD-Sud (*L. reuteri*)	0	17	1	5	5	1	24	70	5

NB:

- High number of hit(s) in isolate, relative to consistent hit range
- Very high number of hits in isolate, relative to consistent hit range

Rf – Riboflavin
Fo – Folate
Ni – Niacin
Th – Thiamine
Amy – Amylase
AMR – Antimicrobial Resistance
Tyr – Tyrosine 2,3 – aminomutase
Trp – Tryptophan
Lyd – Lysine dehydrogenase
Numbers = number of predictive gene hits

5.3.2 Technological properties of LAB isolates

5.3.2.1 Rate of acidification of LAB isolates

At the start of fermentation, the pH of the fermenting substrate inoculated with each of the 27 isolates ranged from 6.04 to 6.07. This however reduced gradually as the fermentation proceeded and by 12 h, they ranged from 3.29 to 5.34 with 55.5 % of the isolates reaching pH levels between 3 and 4 with most of them being isolates of *L. pontis*. The control ranged from 6.06 to 5.44 from start to finish (12 h). The changes in pH at the different intervals is presented in Figure 17a. The titratable acidity on the other hand increased as the fermentation progressed. They increased from 0.06 to 0.09 at the start of fermentation to 0.27 to 0.39 by the end of 12 h fermentation as shown in Figure 17b. Even though few of the isolates recorded very low rate of acidification more than half recorded good rates within the 12 h fermentation period. The three highest change in pH all occurred within 4-8 h. These were 1.79 for *L. fermentum* LMAN-Sdb, 1.54 for *L. reuteri* LDOD-Sud and 1.50 for *L. pontis* LTAD-12g.

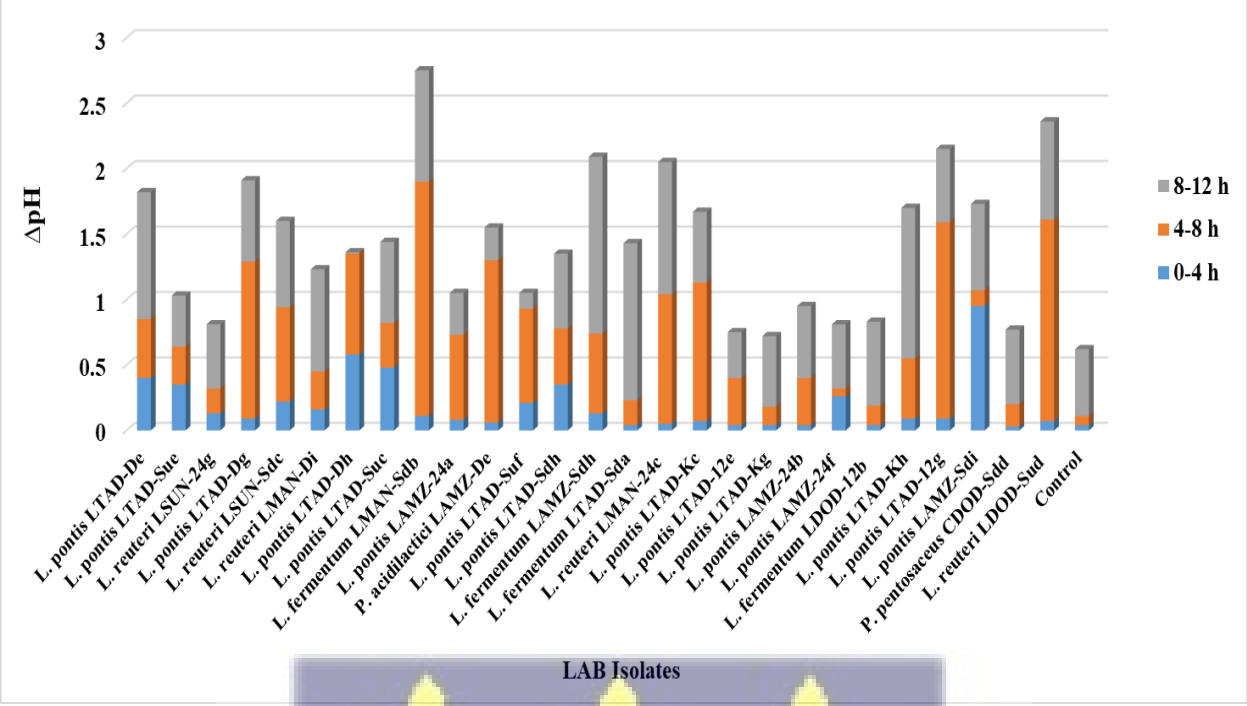


Figure 17a: Change in pH from 0-4 h, 4-8 h, and 8-12 h and control.

Figure 17b: Change in Titratable acidity from 0-4 h, 4-8 h, and 8-12 h.

5.3.2.2 Tolerance of LAB isolates to bile salts

The tolerance of the 27 LAB isolates to different concentrations of bile salts (0.3 %, 0.5 % and 1 %) on MRS agar plates and low pH (1.5, 2.5, 3.5, 4.5 and 6) are presented in Table 11. Almost all the isolates (96.3 %) exhibited good tolerance to bile salts. *L. reuteri* LMAN-Di was the only LAB isolate which was unable to grow even at the lowest concentration of bile salts (i.e 0.3 %). Precipitations had formed on some of the colonies on MRS agar plates.

5.3.2.3 Tolerance of LAB isolates to low-neutral pH

None of the 27 LAB isolates tested could grow at the lowest pH of 1.5 which was tested as seen in Table 11. Sixteen (16) out of the 27 LAB isolates representing 59.3 % showed partial to good growth in pH 2.5. All 27 isolates showed very good tolerance to pH 3.5 to pH 7, the highest pH tested, as they exhibited good growth in that pH range.

Table 11: Bile and pH tolerance of LAB isolates

LAB Isolates	Bile Tolerance			pH Tolerance					
	0.3 %	0.5 %	1.0 %	pH 1.5	pH 2.5	pH 3.5	pH 4.5	pH 6	pH7
L. pontis LTAD-De	+	+	+	-	-	+	+	+	+
L. pontis LTAD-Sue	+	+	+	-	±	+	+	+	+
L. reuteri LSUN-24g	++	+	+	-	+	+	+	+	+
L. pontis LTAD-Dg	++	++	++	-	-	+	+	+	+
L. reuteri LSUN-Sdc	+	+	+	-	±	+	+	+	+
L. reuteri LMAN-Di	-	-	-	-	-	+	+	+	+
L. pontis LTAD-Dh	++	+	+	-	-	+	+	+	+
L. pontis LTAD-Suc	+	+	+	-	-	+	+	+	+
L. fermentum LMAN-Sdb	+	+	+	-	+	+	+	+	+
L. pontis LAMZ-24a	+	+	+	-	-	+	+	+	+
P. acidilactici LAMZ-De	++	++	+	-	-	+	+	+	+
L. pontis LTAD-Suf	+	+	+	-	-	+	+	+	+
L. pontis LTAD-Sdh	+	+	+	-	-	+	+	+	+
L. fermentum LAMZ-Sdh	+	+	+	-	+	+	+	+	+
L. fermentum LTAD-Sda	+	+	+	-	+	+	+	+	+
L. reuteri LMAN-24c	+	+	+	-	+	+	+	+	+
L. pontis LTAD-Kc	++	++	++	-	+	+	+	+	+
L. pontis LTAD-12e	++	+	+	-	+	+	+	+	+
L. pontis LTAD-Kg	+	+	+	-	+	+	+	+	+
L. pontis LAMZ-24b	++	+	+	-	±	+	+	+	+
L. pontis LAMZ-24f	++	++	+	-	+	+	+	+	+
L. fermentum LDOD-12b	++	++	++	-	±	+	+	+	+
L. pontis LTAD-Kh	+	+	+	-	±	+	+	+	+
L. pontis LTAD-12g	++	++	+	-	+	+	+	+	+
L. pontis LAMZ-Sdi	+	+	+	-	-	+	+	+	+
P. pentosaceus CDOD-Sdd	+	+	+	-	+	+	+	+	+
L. reuteri LDOD-Sud	++	++	++	-	-	+	+	+	+

NB: - = No growth; ± = Partial growth; + = Prolific growth; ++ = Growth with precipitation

5.3.2.4 Amylase, protease and exopolysaccharide production by LAB isolates

Amylase, protease and exopolysaccharides production by the LAB isolates are presented in Table 12. The amylolytic potential of the selected LAB isolates tested showed varying degrees of activity. Of 27 isolates tested, 18 (66.7 %) produced clear zone lengths of <1.5 mm to >3 mm. Out

of the 18 isolates that showed clear zones, 12 (66.7 %) were *L. pontis*, 3 (16.7 %) were *L. fermentum*, and 1 (5.6 %) each of *P. acidilactici*, *P. pentosaceus*, and *L. reuteri*. The rest of the isolates did not show amylase activity. None of the 27 isolates showed protease activity, whilst a third of the ioslates produced slime, indicating the production of exopolysaccharides. Six out of the 9 LAB isolates which produced expolysaccharides were *L. pontis* isolates.

Table 12: Amylase, protease and EPS production by LAB isolates

LAB Isolates	Amylase	Protease	EPS
***L. pontis* LTAD-De**	-	-	-
***L. pontis* LTAD-Sue**	-	-	-
***L. reuteri* LSUN-24g**	-	-	-
***L. pontis* LTAD-Dg**	+++	-	++
***L. reuteri* LSUN-Sdc**	-	-	-
***L. reuteri* LMAN-Di**	-	-	-
***L. pontis* LTAD-Dh**	++	-	+
***L. pontis* LTAD-Suc**	+	-	-
***L. fermentum* LMAN-Sdb**	++	-	++
***L. pontis* LAMZ-24a**	+	-	++
***P. acidilactici* LAMZ-De**	+++	-	++
***L. pontis* LTAD-Suf**	+	-	-
***L. pontis* LTAD-Sdh**	++	-	-
***L. fermentum* LAMZ-Sdh**	+++	-	-
***L. fermentum* LTAD-Sda**	+++	-	-
***L. reuteri* LMAN-24c**	-	-	-
***L. pontis* LTAD-Kc**	+++	-	+
***L. pontis* LTAD-12e**	++	-	++
***L. pontis* LTAD-Kg**	+	-	+
***L. pontis* LAMZ-24b**	+	-	-
***L. pontis* LAMZ-24f**	+	-	-
***L. fermentum* LDOD-12b**	-	-	+
***L. pontis* LTAD-Kh**	-	-	-
***L. pontis* LTAD-12g**	+++	-	-
***L. pontis* LAMZ-Sdi**	-	-	-
***P. pentosaceus* CDOD-Sdd**	+	-	-
***L. reuteri* LDOD-Sud**	+++	-	-

NB: - = No clear zone/slime formation; + = Clear zone/slime length of <1.5 mm; ++ = Clear zone/slime length of 1.5 -3.0 mm; +++ = Clear zone/slime length of >3 mm

5.3.2.5 Antimicrobial activity of LAB isolates

Antimicrobial activity of the selected LAB isolates screened against Gram-positive and Gram-negative pathogens (*Salmonella enterica sv typhimurium* Lt2, *Bacillus cereus* VLAG 699*; Enterococcus faecium* ATCC 6057*; Staphylococcus aureus* FI10739*; Micrococcus luteus* FI10640*; Enterococcus faecalis* FI9187 and *E. coli* RMEC0157 NCCBI 100282), showed a wide range of inhibition against these pathogens. Generally, the inhibition activity against all the indicator organisms were moderate to strong, with the exception of *Micrococcus*, where only 11.1 % of the isolates showed moderate inhibition, 85.2 % showed weak inhibition and 3.7 % showed no inhibition at all. Out of the 27 LAB isolates carrying the bacteriocin gene, only *L. reuteri* LMAN-Di and *L. pontis* LTAD-Suc did not have inhibition against 5 and 2 out of 7 pathogens, respectively. Thus largely, the bacteriocin-producing genes of these isolates were fully expressed, making the pathogens tested susceptible to the bacteriocins produced (Table 13).

Table 13: Antimicrobial studies on LAB isolates against indicator organisms

LAB Isolates	Indicator Organisms						
	ST	BC	EF	SA	ML	EFL	EC
L. pontis LTAD-De	+++	+++	+++	++	+	++	+
L. pontis LTAD-Sue	+++	+	++	+	+	++	++
L. reuteri LSUN-24g	++	++	+++	+	++	++	+++
L. pontis LTAD-Dg	+++	+	+++	++	++	++	+++
L. reuteri LSUN-Sdc	+++	+	++	+	++	++	++
L. reuteri LMAN-Di	-	-	++	-	-	-	+
L. pontis LTAD-Dh	++	+	++	++	+	+	+++
L. pontis LTAD-Suc	++	-	+	+	+	-	+++
L. fermentum LMAN-Sdb	+	+	++	++	+	++	++
L. pontis LAMZ-24a	+++	++	+++	+++	+	+++	++
P. acidilactici LAMZ-De	++	++	+++	+++	+	+++	++
L. pontis LTAD-Suf	++	++	+++	+++	+	+++	++
L. pontis LTAD-Sdh	+	+++	+++	+++	+	+++	++

L. fermentum LAMZ-Sdh	+++	+++	+++	+++	+	+++	+++
L. fermentum LTAD-Sda	+++	+++	+++	+++	+	+++	++
L. reuteri LMAN-24c	+++	+++	+++	+++	+	+++	+++
L. pontis LTAD-Kc	+++	+++	+++	+++	+	+++	+++
L. pontis LTAD-12e	+++	+++	+++	+++	+	+++	++
L. pontis LTAD-Kg	+++	+	++	+	+	++	++
L. pontis LAMZ-24b	+++	+++	++	++	+	+++	++
L. pontis LAMZ-24f	+++	+++	+	++	+	++	+
L. fermentum LDOD-12b	+++	+++	++	++	+	+++	+++
L. pontis LTAD-Kh	++	+++	+	+	+	++	+
L. pontis LTAD-12g	+++	+++	++	+	+	+++	++
L. pontis LAMZ-Sdi	+++	+++	++	++	+	+++	+++
P. pentosaceus CDOD-Sdd	+++	+++	++	+	+	+++	++
L. reuteri LDOD-Sud	+++	+++	++	++	+	+++	+++

NB: ST = *Salmonella enterica sv typhimurium Lt2*; BC = *Bacillus cereus VLAG 699*; EF = *Enterococcus faecium ATCC 6057*; SA = *Staphylococcus aureus FI10739*; ML = *Micrococcus luteus FI10640*; EFL = *Enterococcus faecalis FI9187*; EC = *E. coli RMEC0157 NCCBI 100282*

- = No inhibition; + = weak inhibition; ++ = moderate inhibition; +++ = strong inhibition

5.3.3 Technological and probiotic properties of yeast isolates

5.3.3.1 Yeast screened

With the exception of *Clavispora lusitaniae* a known non-beneficial yeast, probiotic potentials were determined for all 53 yeast isolates comprising *S. paradoxus*, *S. cerevisiae*, *P. kudriavzevii* and *C. tropicalis*.

5.3.3.2 Effect of pH

All the yeast isolates were able to tolerate or withstand the low-neutral pH conditions of pH 2.0, 3.0, 5.5 and 7.0. However, at the lower pH of pH 1.5, only strains of *P. kudriavzevii* and *C. tropicalis* were able to grow (Table 14).

5.3.3.3 Effect of bile

All 53 yeast isoates were able to grow in the presence of different concentrations of bile salts i.e 0.3 %, 0.5 %, 1.0 % (Table 14).

5.3.3.4 Effect of different temperatures

All the yeast isolates were able to grow well at 25 °C and 37 °C, however at 42 °C, only strains of *P. kudriavzevii* and *C. tropicalis* grew prolifically. Approximately 41.2 % of *S. cerevisiae* strains tolerated the high temperature of 42 °C whilst the remaining 58.8 % did not grow at all. *S. paradoxus* strains did not grow at the higher temperature of 42 °C (Table 14). The strains *of P. kudriavzevii* produced fruity aroma during the fermentation trials.

5.3.3.5 Effect of salt concentration

All the yeast isolates were able to grow at salt concentrations of 4 and 6 % NaCl, however the effect on the yeast isolates were strong beyond these concentrations. Only strains of *C. tropicalis* grew well in the presence of 10 % NaCl while two strains of *S. cerevisiae* (*S. cerevisiae* YDOD-Suc and *S. cerevisiae* YMAN-Sdc) grew partially. There was no growth by any of the strains tested in 20 % NaCl (Table 14).

Table 14: Tolerance of yeast isolates to low pH, bile, temperature and salt

Yeast Isolates	pH Tolerance					Bile Tolerance (%)			Temperature Tolerance (°C)			Salt (NaCl) Tolerance (%)			
	1.5	2	3	5.5	7	0.3	0.5	1	25	37	42	4	6	10	20
S. cerevisiae YTAD-12g	-	+	+	+	+	+	+	+	+	+	±	+	+	-	-
S. cerevisiae YSUN-Kf	-	+	+	+	+	+	+	+	+	+	±	+	+	-	-
S. cerevisiae YTAD-De	-	+	+	+	+	+	+	+	+	+	±	+	+	-	-
P. kudriavzevii YTAD-Kh	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
S. cerevisiae YSUN-24i	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-

S. cerevisiae YSUN-Kj	-	+	+	+	+	+	+	+	+	+	±	+	+	-	-
C. tropicalis YTAD-Da	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
P. kudriavzevii YTAD-12j	+	+	+	+	+	+	+	+	+	+	+	+	+	±	-
S. cerevisiae YMAN-24d	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. cerevisiae YDOD-Sda	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
P. kudriavzevii YAMZ-Sug	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
S. cerevisiae YDOD-Sdb	-	+	+	+	+	+	+	+	+	+	±	+	+	-	-
P. kudriavzevii YMAN-Kg	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
S. cerevisiae YMAN-Sdc	-	+	+	+	+	+	+	+	+	+	-	+	+	±	-
P. kudriavzevii YAMZ-Kh	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
S. cerevisiae YSUN-Sud	-	+	+	+	+	+	+	+	+	+	±	+	+	-	-
C. tropicalis YDOD-Suh	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
S. cerevisiae YSUN-Suf	-	+	+	+	+	+	+	+	+	+	±	+	+	-	-
S. cerevisiae YMAN-Sud	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
P. kudriavzevii YTAD-Sdc	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
S. cerevisiae YMAN-Sue	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. cerevisiae YAMZ-Sdb	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
P. kudriavzevii YTAD-Kg	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
P. kudriavzevii YMAN-Kc	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
P. kudriavzevii YTAD-Suf	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
S. cerevisiae YDOD-Suc	-	+	+	+	+	+	+	+	+	+	-	+	+	±	-
S. cerevisiae YTAD-12f	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. cerevisiae YSUN-Sda	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YDOD-12j	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YMAN-24g	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YAMZ-Kb	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YTAD-Kd	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
C. tropicalis YDOD-Ke	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
S. paradoxus YAMZ-Sdc	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YAMZ-Da	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YDOD-12e	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YSUN-24h	-	+	+	+	+	+	+	+	+	+	±	+	+	-	-
S. paradoxus YTAD-Sud	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YDOD-Sud	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YSUN-Sdi	-	+	+	+	+	+	+	+	+	+	±	+	+	-	-
S. paradoxus YDOD-Sdg	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YAMZ-Db	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YDOD-Dc	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YSUN-24a	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YDOD-12g	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YSUN-Sdb	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YMAN-24j	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YSUN-24b	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-

S. paradoxus YAMZ-24i	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YTAD-12a	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YTAD-12b	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YDOD-Sua	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
S. paradoxus YAMZ-Ka	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-

NB: - = No growth; ± = Weak growth; + = Profuse growth

5.4 Discussion

Limosilactobacillus fermentum LMAN-Sdb, *L. pontis* LTAD-12g, *L. reuteri* LDOD-Sud, and *L. fermentum* LAMZ-Sdh were the isolates which exhibited the fastest rates of acidification. Their use as starter culture to produce *Hausa koko* is therefore likely to reduce the duration for the fermentation of millet slurry which is significant for starter culture development based on outcomes from further probiotic assessments. This acidification stage results in the production of organic acids by the LAB which reduces the pH of the fermenting substrate. This also imparts antimicrobial effect by interfering with the maintenance of cell membrane potential, reducing intracellular pH, inhibiting active transport and inhibiting a variety of metabolic functions (Ross et al., 2002). The organic acids act as preservatives in foods due to the broad spectrum of their antimicrobial inhibitory properties and as acidulants. They can decrease the growth of other microorganisms by reducing the pH of the food matrix to a point that inhibits microbial growth (Hinton, 2006; Plumridge et al., 2004; Steiner & Sauer, 2003; Dziezak, 1990). They impart stabilizing and preservation properties on the food in addition to flavour enhancement. These are in addition to their other qualities which ultimately improves the overall sensory attributes of the food (Min et al., 2007; Gomis 1992). Though *P. kudriavzevii* was not one of the isolates which exhibited the fast rates of acidification, it produced a desirable fruity aroma during the fermentation trials and will improve the sensory quality of *Hausa koko* if it is incorporated into starter culture mix. The desirable sensory attribute of *P. kudriavzevii* has been reported by Holt et al., (2018) who used it in beer production and attributed its desirable sensory quality to the production of flavor-active compounds including esters, high alcohol concentration and phenolic compounds.

All the *L. pontis, L. fermentum* and *L. reuteri* isolates contained genes predicted to encode the bacteriocin Enterolysin A, although this bacteriocin have mainly been reported from *Enterococcus*

faecalis species (Šušković et al., 2010). A similar outcome has been reported in the genome of *L. fermentum* species from selected fermented foods including *Ogi* from Nigeria using BAGEL 3 database and BLASTP (Abdulkarim et al., 2020). Other isolates including *L. plantarum* showed genes predicted to encode plantaricin structural protein, LanM lantibiotic, ATP binding protein and plantaricin immunity protein (Abdulkarim et al., 2020). *Pediococcus acidilactici* LAMZ-De genome showed genes for Mersacidin and Enterolysin A structural proteins. Mersacidin is the smallest lantibiotics which contains lan thionine antibiotics with activity against cell wall peptides. This bacteriocin also inhibits the growth of pathogens (Sass et al., 2008). Its production has also been reported in *Bacillus* strains (Viel et al., 2021). The CDOD-Sdd (*P. pentosaceus*) genome showed a putative Bovicin 255 and Penocin A structural protein. Penocin A is a pediocin-like bacteriocin which was originally produced from *Pediococcus pentosaceus* and forms part of the class IIa bacteriocins. They have narrow spectrum of activity against pathogens by inducing pore formation in the cells resulting in their death (Jiang et al., 2021; Collins et al., 2018; Cotter et al., 2005). Bovicin 255 are regarded as nonlantibiotics classified as class II bacteriocins identified in the gut of ruminant associated *Streptococcus* strains including *bovis, equinus* and *gallolyticus* (Garsa et al., 2019; Gilbert, 2016). It also has activity against certain strains of foodborne indicators (McAllister et al., 2011). The use of genomic data is making it easier to identify and predict bacteriocin encoded genes within the genome of an isolate even though these genes may not always translate into antimicrobial activity due to several factors (Collins et al., 2018). Olasupo et al. (1999) also reported the occurrence of nisin Z produced by *Lactococcus lactis* BFE 1500 from an indigenous cheese product from Nigeria, which inhibited not only some LAB but also other pathogenic bacteria. Bacteriocin production has been reported in other LAB strains from cereal fermented foods including maize flour during *doklu* production (Assohoun-Djeni et al., 2016). It

is therefore necessary to probe further on the ability of these isolates to actually inhibit pathogens and indicator organisms.

Exhibition of such antibacterial activity by the LAB is one of the key benchmarks for selecting a potential probiotic isolate for starter culture development. Such isolates will improve the safety of *Hausa koko* due to their antimicrobial activity against both Gram-positive and Gram-negative pathogens. Bacteriocins produced by LAB have several characteristics that makes them ideal for utilisation as food preservatives. They are nontoxic and inactive against eukaryotic cells and have minor effect on the gut microflora (Theron & Lues, 2010). They are primary metabolites due to their simple biosynthetic mechanisms when compared to conventional antibiotics which are secondary metabolites, thus it is much easier to increase their activity and/or specificity towards target microorganisms through bioengineering (Perez et al., 2014). They are therefore used to control the growth of pathogens at various industrial food systems (McEntire et al., 2003). Some of the food spoilage and pathogenic microorganisms sensitive to bacteriocins in relation to fermentation are *Enterococcus, Staphylococcus* spp., *Listeria monocytogenes, Clostridium* and *Bacillus spp* (Rattanachaikunsopon & Phumkhachorn, 2010). In the gastrointestinal tract (GIT), bacteriocins may act as helper peptides for the available probiotic strains to destroy susceptible pathogens (López-Cuellar et al., 2016).

About half of the LAB isolates produced very little or no amylase enzyme while the other half showed substantial amylase activity. This pattern appeared not to be strain specific as for the same species different isolates produced none or varying amounts of the enzyme. The other half of the LAB isolates produced substantial amounts of amylase. In cereal fermentation, amylase is the key enzyme used in saccharification of starch yielding fermentable sugars (Egwim & Oloyede, 2006). However, cereals are usually malted before they produce substantial levels of amylase or diastatic

activity as occurs in the production of some of the local non-alcoholic beverages such as *Nmeda* and *Asaana* as well as alcoholic beverages, such as *pito* and *burukutu*. That notwithstanding, the amylolytic potential of LAB isolated from different fermented foods have been reported by Sanni et al., (2002). Xu et al., (2020) reported the amylolytic and probiotic potential of LAB from Chinese fermented cereal based foods. These isolates demonstrated tolerance to low pH (2 and 3), bile (0.3 and 0.6 %), antimicrobial activities against pathogens and high auto-aggregation. They consequently suggested its usage for starter cultures to improve the fermentation process. The use of the amylolytic LAB from *Hausa koko* in the development of starter culture for the product is very relevant. These strains will aid in the hydrolysis of starch from millet which will help in releasing of nutrients (Oguntoyinbo & Narbad, 2012). Songré-Ouattara et al., (2009) reported the use of amylolytic LAB to hydrolyse starch in order to increase the energy density in pre-cooked pearl millet gruel. According to Motarjemi & Nout, (1996), the viscosity of starchy gruels may be reduced by amylolytic LAB during fermentation thereby improving the nutrient content and at the same time maintaining an acceptable semi-solid consistency or thickness of the gruel.

Production of exopolysaccharides (EPS) by the 27 LAB isolates were very low (33.3 %). Out of this, only the species *L. pontis* (66.7 %), *L. fermentum* (22.2 %) and *P. acidilactici* (11.1 %) demonstrated EPS production. Production of EPS by LAB starter cultures is valued in cereal fermented foods as they impact on their textural properties (Owusu-Kwarteng et al., 2015). In milk fermentation, production of exopolysaccharides has been reported to improve its physical properties acting as gelling agent/emulsifier, viscosifier and stabilizer providing the product with its natural thickness and improving its flavouring and sensory characteristics greatly (Behare et al., 2009; Ruas-Madiedo & Reyes-Gavilan, 2005).

The quest for functional foods by the growing population has boosted interest in the benefit of EPS synthesized from *Limosilactobacillus* species on the health of consumers (Ruas-Madiedo et al., 2002). EPS from LAB lowers blood cholesterol level and possess anticanceral and antitumoral activities (Sanalibaba & Çakmak, 2016; Behare et al., 2009). Dextran from *L. fermentum*, Mutan from *L. reuteri* ML1, Reuteran from *L. reuteri* 121 which are all used in the food industry are some common polysaccharide EPS produced by *Limosiactobacillus* species. However, their usage industrially is hindered by the high cost of production and low yield (Oleksy & Klewicka, 2018).

Some of the stressful conditions or challenges potential probiotic strains must overcome in the GIT are low pH and bile. With the exception of pH 1.5, the majority of LAB isolates exhibited good tolerance to low pH levels (2.5, 3.5, 4.5, 6.0 and 7.0) although a few strains grew partially or did not grow at all in pH 2.5. Similarly, all the yeast isolates showed great tolerance to the different pH conditions (1.5, 2.0, 3.0, 5.5 and 7.0), with the exception of pH 1.5, where only *P. kudriavzevii* and *C. tropicalis* strains survived. These outcomes from both LAB and yeast isolates can be attributed to their high tolerance and survival in acid conditions in general, given the fact that normal gastrointestinal pH in the stomach ranges from 1.5 - 3.5 (Marieb & Hoehn, 2018). Koziolek et al., (2015) reported fluctuating mean pH values of 1.7 - 4.7 during gastric transit study in the fasting state in humans. These further increased from 5.9 - 6.3 in proximal parts to 7.4 - 7.8 in distal parts during small bowl transit. Other reports also claim during fasting periods, acidity in the stomach is about 1.5 but increases to 4.5 after meals (Owusu-Kwarteng et al., 2015). This suggest that surviving isolates from this study are potential probiotic strains. They can withstand the organic acidic condition from their own metabolism in the fermented millet (Owusu-Kwarteng et al., 2015) as well as the hydrolic acid concentrations in the stomach as it is the main obstruction for thriving microorganisms in the stomach (Psomas et al., 2001).

LAB and yeast strains also exhibited good tolerance to the antimicrobial agent, bile, at different concentrations (0.3, 0.5 and 1.0). The mean bile concentration in the intestinal tract is 0.3 % (w/v) and the time food stays in the small intestine is estimated at 4 h (Prasad et al., 1998). The LAB and yeast species tolerance against the different bile concentrations within 24 - 48 h is an indication of their survival abilities to bile content in the intestinal tract affirming their probiotic potential (Psomas et al., 2001). The precipitations on some of the LAB strain colonies may be the production of bile salt hydrolase which were not confirmed in this study. Generally, all the yeast isolates exhibited strong tolerance to high temperatures of 37 °C, which according to Gil-Rodríguez et al., (2015) is a desired temperature for potential probiotic strains to survive the host temperature and for propagation purposes. These yeast strains can therefore survive during propagation in temperatures prevailing in Ghana and most parts of Africa. While only some strains of *S. cerevisiae* exihibited partial growth at extreme temperatures of 42 °C, all the *P. kudriavzevii* and *C. tropicalis* strains survived with good growth. Strong tolerance to salt conditions at 4 % and 6 % is a good indication but their poor tolerance at 10 % and 20 % as observed in this study agrees with reports by AbdElatif et al., (2016) that the survival of *S. cerevisiae* is affected even at 6 % NaCl. High tolerance to low-neutral pH, bile salts, temperatures up to 37 °C are among the essential criteria for strain selection as probiotics that should survive the conditions present in the gastrointestinal tract and compete favourably with other microorganisms (Mokoena et al., 2016; García-Hernández et al., 2012; Rajkowska & Kunicka-Styczynska, 2010). These therefore makes the tested isolates in this study potential probiotics.

The results of the present study on the probiotic potential of lactic acid bacteria and yeast strains are similar to reports from other cereal (including millet) fermentations. In a study to select potential probiotic cultures, Greppi et al., (2017) reported that out of 93 yeast strains, 99 %

tolerated 0.3 % bile salt concentration, whilst 31 % tolerated pH 2 and between 11- 45 % tolerance after human digestion simulation. The best performing yeast strain with good probiotic potential according to Greppi et al., (2017) was *Pichia kudriavzevii*. The predominant *L. fermentum* strains isolated from fermented millet dough associated with *fura* processing were confirmed by Owusu-Kwarteng et al., (2015) to have potential application as probiotic starter cultures. Even though the yeast population reported in *fura* by Pedersen et al., (2012) were totally different from those identified and characterised in this study, their survival in pH 2.5, 0.3 % bile salts and growth at 37 °C was interpreted as indication of their survival during passage through the interstinal tract. They also reported the effect on their transepithelial electrical resistance (TEER). Ogunremi et al., (2015) reported of yeast strains from cereal based traditional fermented Nigerian foods (*ogi*, *kunu-zaki* and *burukutu*) which had potential probiotic properties with growth populations after 24 h in the range of 7 - 8 log CFU/ml. These strains maintained viability at 37 °C, survived in pH 2.0 and 2 % bile salts. Additionally, they were able to display lipase, protease and phytase activity as well as removal of cholesterol.

The probiotic potentials of the species studied in this work are similar to those reported in other studies. Owusu-Kwarteng et al., (2015) investigated the probiotic potential of sixteen different strains of *L. fermentum* isolates from fermented millet dough in *fura* production and reported their desirable probiotic and technological features as well as their potential use as starter cultures. Lei & Jacobson (2004) also tested the antimicrobial activities as well as the resistance of LAB isolates from *koko* and *koko* sour water (KSW) to low pH and bile salts. Further clinical studies on the LAB isolates from KSW confirmed their probiotic potential in the treatment of young children with diarrhoea (Lei et al., 2006). LAB species including *L. plantarum, L. lactis* and *L. fermentum* from *kunu-zaki* beverages were identified as potential probiotics that could be used in human

preparations (Oluwajoba et al., 2013). A number of clinical studies have proven the probiotic prospects of *L. reuteri* in treating gastrointestinal tract conditions like diarrhoea and other infections (Indrio et al., 2014; Gutierrez-Castrellon et al., 2014; Francavilla et al., 2014; Weizman et al., 2005).

A faster rate of acidification and demonstration of other good technological and probiotic potentials was finally used as the key criteria for selection of isolates for starter culture development. Based on these criteria *L. fermentum* LMAN-Sdb, *L. reuteri* LDOD-Sud and *L. pontis* LTAD-12g were selected for starter culture trials in a subsequent study. With respect to yeasts, the probiotic properties of *S. cerevisiae* and *S. paradoxus* were similar but *S. cerevisiae* was preferred because of its better tolerance to environmental stress as reported by Warringer et al., (2011). Again, *S. cerevisiae* is used in several fermented foods globally and also used as a probiotic. The selected yeast isolates that showed good probiotic characteristic were *S. cerevisiae* YSUN-Sud and *P. kudriavzevii* YTAD-12j.

5.5 Conclusion

Technological properties as well as probiotic characteristics of LAB and yeast isolated during *Hausa koko* production have been assessed in this study. Strain-specific differences existed between the different LAB and yeast species with respect to their technological and probiotic properties. Most strains exhibited good and promising characteristics and may contribute to the quality and safety of *Hausa koko*. Out of these, three LAB isolates, *L. reuteri* LDOD-Sud, *L. pontis* LTAD-12g and *L. fermentum* LMAN-Sdb, and two yeast isolates, *S. cerevisiae* YSUN-Sud and *P. kudriavzevii* YTAD-12j, were selected for further studies in the development of a starter culture

for *Hausa koko.* The probiotic properties of *S. cerevisiae* and *S. paradoxus* were similar but *S. cerevisiae* was preferred because it has a better tolerance to environmental stress and is widely used in several fermented foods globally and also as probiotic yeast. The results of the *in-vitro* test strongly suggest that these strains are potential probiotics due to the expression of some key technological and probiotic attributes. However, these isolates should be evaluated further for their development as a starter culture for the production of *Hausa koko*.

CHAPTER SIX

6.0 Starter culture development

6.1 Introduction

Historically, production of fermented foods globally was spontaneously performed by the indigenous microbiota from the raw materials, contact surfaces, or the environment. But this practice is changing with advancement in genetic engineering technologies used in starter culture selection and emergence of controlled large-scale fermentations (bioprocess technologies) in advanced countries (De Melo Pereira et al., 2018). However, the narrative is different in most African countries where fermentation is still largely done spontaneously using rudimentary traditional methods which are often unhygienic, laborious, time consuming and at house-hold levels. Several challenges are associated with these indigenous unpredictable and uncontrolled food fermentation processes such as poor microbial quality, safety, prolonged fermentation time, short shelf life and undesirable sensory attributes (Gadaga et al., 2004). A significant challenge with cereals fermentation is their contamination with mycotoxins, bacterial toxins, cyanogenic glycosides and biogenic amines (Sivamaruthi et al., 2019; Kpodo et al., 1996). Mycotoxins are fungal secondary metabolites produced by toxigenic strains of *Aspergillus*, *Fusarium* and *Penicillium* (Terzi et al., 2014). Mycotoxins may contaminate cereals through poor handling making them prone to contamination. These contaminations may occur in the field, during harvesting, processing, transportation or storage (Wang & Xie, 2020). Aflatoxins, ochratoxins, patulin, fumonisins, trichothecenes and zearalenone are the six main mould producing mycotoxins affecting foods. *Aspergillus flavus* and *Aspergillus parasiticus* are the toxigenic fungi responsible for aflatoxin B1, B2, G1, G2, M1 and M2 production in cereals with B1 being the most potent.

Their contamination affects commercial trade globally. Most importantly, their occurrence in food systems is undesirable and dangerous, causing nutritional losses and poses public health concerns as they can cause adverse health effects including liver cancer. Nevertheless, their presence may be curbed by antifungal activity during LAB fermentation (Wang & Xie, 2020; Marin et al., 2013; Amaike & Keller, 2011; Dalié et al., 2010; Reddy et al., 2010; Gerez et al., 2009; Diener et al., 1987).

Other challenges associated with cereal fermentation such as product failure, antinutrients, quality and safety can also be mitigated by the use of starter cultures with potential probiotic features during the fermentation process (Ogunremi et al., 2017; Kimaryo et al., 2000). Starter cultures are preparations or material containing high populations of strains of one or more viable microorganisms (bacteria, yeast and/or moulds or their combination) with stable features, which are added to initiate and accelerate the fermentation process (Sulieman, 2017; Wakil et al., 2014; Holzapfel, 2002 & 1997). These strains can be selected from the microflora of indigenous fermented complexes. The selected strain(s) has an important influence on the characteristics of the final fermented product they are used in (Manini et al., 2016). They take control of the initial phase of a fermentation process when they adapt well to the substrate (Holzapfel, 2002). They are usually categorised as single strain containing only one strain per specie, multi-strains containing three or more single strains of well-defined mixtures, and mixed strains that contain unknown strains (Mishra et al., 2017). They are also categorised based on growth temperature (mesophilic and thermophilic starter cultures), flavor production ability and type of end products resulting from glucose metabolism (homo or hetero fermenters) (Sulieman, 2017). Cautious selection of such microorganisms with probiotic features is key. Fast secretion of inhibitory metabolites, antimicrobial activities, resistance to stressful conditions, sensory modifications amongst others

are some of the most important consideration criteria for lactic starter culture development (De Melo Pereira et al., 2018; Zoumpopoulou et al., 2017; Marchesi et al., 2016; Alfonzo et al., 2013). Their application produces products with expected outcomes that are reproducible with improved nutritional, sensory, functional and most importantly safety qualities (Ogunremi et al., 2017).

In the past few decades several studies have been carried out in various African countries to study the microbiology and biochemistry of indigenous fermented foods. Such studies have focused primarily on the dominant microorganisms associated with the fermentation processes. However, some studies have focused on the development of starter culture for the otherwise traditional fermentations using LAB and yeast mostly (Greppi et al., 2017; Annan et al., 2015; Akabanda et al., 2014; Fadahunsi et al., 2012; Ali & Mustafa, 2009; Halm et al., 1996). One such category of fermented foods in high demand globally are fermented cereal foods (Petrova & Petrov, 2020) even though they are mostly spontaneously fermented especially in developing countries. Although indigenous fermentation of cereals generally improves their safety, nutritional qualities, taste, appearance and other sensory attributes, there is an increasing demand for faster delivery of fermented cereal foods of superior stability, health benefit, consistency, quality and safety (Anal, 2019; Gille et al., 2018; Ogunremi et al., 2017; Ferri et al., 2016). These demands are because of the awareness of the benefits from healthy foods by consumers, globalisation, increasing international trade and travels, transformations in eating habits and many more. There is an urgent need to guarantee the quality and safety of indigenous fermented foods in general, not only for local consumers but also for the international community (Soro-Yao et al., 2014). This can be achieved using potential probiotic starter cultures. The usage of starter culture on indigenous populations in fermented foods would accelerate the fermentation process tremendously (Solieri et al., 2013).

Currently, there is very little information on the use of starter culture for the fermentation of millet in the international literature though there are several publications on microbial species involved in the traditional fermentation of millet. *Hausa koko*, a popular breakfast porridge is very widely consumed in Ghana and several other African countries, but its production has largely remained traditional, even at the Small and Medium Enterprises (SMEs) level depending upon spontaneous fermentation of the millet. This study was carried out to develop a starter culture for the fermentation of millet during *Hausa koko* production using probiotic cultures of lactic acid bacteria and yeasts isolated from traditional spontaneous fermentation of millet during *Hausa koko* production.

6.2 Materials and Methods

6.2.1 Selected isolates

A total of 500 LAB and 250 yeast cultures were isolated from *Hausa koko* samples; 90 successfully sequenced LAB were pre-screened using BAGEL4 and PATRIC v3.6.2 before selecting 27 beneficial LAB for further screening for their technological and probiotic properties. For yeast isolates, 58 were successfully sequenced but 53 were selected for further screening for their technological properties as the rest (5) were considered non-beneficial. After testing, three LAB isolates, *Limosilactobacillus fermentum* LMAN-Sdb (F), *Limosilactobacillus reuteri* LDOD-Sud (R), and *Limosilactobacillus pontis* LTAD-12g (P), and two yeast isolates, *Saccharomyces cerevisiae* YSUN-Sud (C), *Pichia kudriavzevii* YTAD-12j (K) were selected for starter culture trials. The isolates were stored at -80 ºC in 40 % glycerol for the starter culture development.

6.2.2 Antimicrobial interactions

The selected LAB and yeast were tested for antimicrobial interactions between the isolates using the agar well diffusion method (Olsen et al., 1995; Schillinger & Lücke, 1989). Circular wells were made using sterile cork borer into solidified appropriate agar (MRS agar for LAB or Malt Extract agar (MEA) for yeast) in Petri plates in duplicates. Within each well 100 µl of overnight cultured LAB or yeast was added and allowed to diffuse into the agar for 5 h. The wells were then overlaid with the appropriate (MRS or MEA) 10 ml sterile soft agar (0.7 % agar) containing 100 µl of the matching overnight cultured indicator LAB or yeast strain. The plates were then incubated at 30 ºC for 48 h and observed for clearing zones. Negative control contained sterile broth or agar without cultures.

6.2.3 Millet flour

Early millet variety known as *Waapp-naara* obtained from CSIR-Savanna Agriculture Research Institute (SARI) was selected for *Hausa koko* production and characterisation of starter culture. The millet was hand sorted, winnowed and milled in a commercial attrition mill. The flour was packaged into polyethylene bags (500 g each), part was irradiated at 5 kGy radiation dose (according to Mustapha et al., 2014; European Food Safety Authority, 2011) at the Ghana Atomic Energy Commission whilst another part was left unsterilized. These were used for the slurry preparations.

6.2.4 Cell harvesting for laboratory inoculum preparation

Selected LAB and yeast cultures were grown overnight by inoculating 100 µl of glycerol stocks in 10 ml MRS (Oxoid CM361) and Malt Extract (Oxoid CM57) broths and incubated at 30 and 25 ºC respectively for 16 -18 h. The culture (100 µl) was transferred into another 10 ml of each respective growth media and incubated appropriately for 16-18 h. The cultures were transferred into falcon tubes, centrifuged at 4000 rpm for 10 min, supernatant discarded and washed with 10 ml sterile Salt Peptone Solution (SPS) of pH 7.2. The wash was repeated three times. The cells were resuspended in 10 ml sterile distilled water for immediate inoculation of millet slurry or stored in Salt Peptone Solution (SPS) kept at 4 ºC for later use (not more than two weeks).

6.2.5 Starter culture fermentations

Multiple (double and triple) strain starter culture fermentation was carried out using irradiated millet slurry (10 % w/w). The slurry was prepared by weighing 20 g of irradiated millet flour and adding 180 g of sterile distilled water to form a 10 % millet substrate. Harvested overnight cultures of LAB and yeast were inoculated to achieve a concentration of 10^7 or 10^6 CFU/ml for LAB or

yeast respectively. The slurry was inoculated with 15 different double and triple combinations of cultures in duplicates. The combinations were:

RP = *L. reuteri* LDOD-Sud + *L. pontis* LTAD-12g;

RF = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb;

RC = *L. reuteri* LDOD-Sud + *S. cerevisiae* YSUN-Sud;

RK = *L. reuteri* LDOD-Sud + *P. kudriavzevii* YTAD-12j;

PK = *L. pontis* LTAD-12g + *P. kudriavzevii* YTAD-12j;

PF = *L. pontis* LTAD-12g + *L. fermentum* LMAN-Sdb;

PC = *L. pontis* LTAD-12g + *S. cerevisiae* YSUN-Sud;

FC = *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;

FK = *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j;

RPC = *L. reuteri* LDOD-Sud + *L. pontis* LTAD-12g + *S. cerevisiae* YSUN-Sud;

RPK = *L. reuteri* LDOD-Sud + *L. pontis* LTAD-12g + *P. kudriavzevii* YTAD-12j;

RFC = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;

RFK = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j;

PFC = *L. pontis* LTAD-12g + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;

PFK = *L. pontis* LTAD-12g + *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j.

Fermentation was carried out after the slurry was inoculated with the starter cultures, vigorously shaken to mix, covered and allowed to ferment for 12 h at room temperature (*ca* 28-30 ºC). Samples were taken every 4 h intervals (0, 4, 8 and 12 h) for determination of pH and microbial (LAB and yeast) counts. pH was taken directly after homogenization using pH meter (MettlerToledo, Switzerland) after calibration with standard buffers. Microbial (LAB and yeast)

populations were enumerated using the pour plate method on deMan, Rogosa and Sharpe (MRS, Oxoid CM359, Oxoid Ltd., Basingstoke, Hampshire, UK.) for LAB and Malt Extract agar (MEA, Oxoid CM0059, Oxoid Ltd., Basingstoke, Hampshire, UK.) for yeast. Uninoculated batch was used as control.

6.2.6 Sample preparation and determination of aflatoxin levels after starter culture fermentation

Different batches and varieties of millet were purchased from the open markets and screened for the presence of naturally aflatoxin contamination until a batch produced positive result containing 23.63 µg/kg of G2; 1.78 µg/kg of B1; 40.14 µg/kg of B2 without aflatoxin G1. The aflatoxin contaminated millet was milled into a flour using a laboratory knife mill (Retsch GM300, Germany). This was used to prepare millet slurries (10 % w/w) by weighing 20 g of aflatoxin contaminated flour and adding 180 g of sterile distilled water for each slurry. Each slurry was inoculated with one of the 15 different combinations of cultures and fermented for 12 h at RT (28-30 ºC). A negative control was also prepared. Their pH was monitored during 12 h fermentation and at the end of 12 h, the samples were shaken vigorously and stored at -20 ºC.

Using the method described by Stroka & Anklam, (1991) for the extraction of aflatoxins in the samples, 50 g was extracted with 200 ml methanol water (80 %) containing 5 g NaCl. The extract was then filtered using Watman Paper No. 4 and diluted with Phosphate Buffered Saline. It was then passed through an immunoaffinity column (R-Biopharm Rhone Ltd. Easi-Extract Aflatoxin) which contained antibodies specific for aflatoxins B1, B2, G1 and G2. The aflatoxins in the samples were then eluted from the immunoaffinity column with pure methanol (100 %). Aflatoxin contents were quantified by HPLC (Agilent 1200 infinity series, Germany) using a laboratory standard containing 2.1 µg/kg of G1; 9.23 µg/kg of G2; 1.95 µg/kg of B1 and 7.64 µg/kg of B2.

6.2.7 Laboratory based sensory analyses of *Hausa koko* produced with starter culture

6.2.7.1 Laboratory preparation of millet porridge and *Hausa koko*

Millet, which was free of aflatoxins and microbiologically safe, was used for the sensory analyses. Two different batches of millet porridge (without spices as *Hausa koko* would) and a batch of *Hausa koko* (millet porridge containing spices) were prepared following the traditional process for the sensory analyses. These batches contained (i) starter culture as inoculum enrichment' that is, starter culture in non-sterile millet flour, (ii) Starter culture and sterile millet flour, (iii) starter culture as inoculum enrichment, that is, starter culture in a modified traditional *Hausa koko* process. In the modified process, millet grains were de-stoned winnowed, washed and de-stoned again in water three times in the laboratory. The millet grains were steamed covered over boiling water in a strainer for 10 min (selected based on microbial analyses outcomes) to reduce the microbial load. It was allowed to cool for 30 min before steeping with water that had been boiled (100 ºC), cooled and inoculated with the respective starter culture combination.

The first batch of millet porridge was prepared without addition of spices using non-irradiated millet flour (10 % w/w) by weighing 40 g of millet flour and adding 360 g of sterile distilled water. It was prepared using the starter cultures as inoculum enrichment, that is, adding the starter culture to unsterilized millet and hence, contained its natural microbiota, which would have been responsible for its spontaneous fermentation. Harvested overnight cultures (15 different double and triple combinations) of LAB and yeast were inoculated into the slurries to achieve a concentration of 10^7 or 10^6 for LAB or yeast respectively and fermented for 12 h at RT (28-30 ºC) with a control, making a total of 16 samples. The fermented millet slurry was cooked into porridge by decanting about 80 % of the supernatant of the fermented slurry into a cooking utensil, 150 ml of water added, boiled and the sediment stirred and added to the boiling water whilst stirring

continuously into a smooth flowing porridge which were assessed by a panel for consumer acceptability.

The second batch of millet porridge was prepared in the same manner but using starter culture and sterile millet flour. The fermented slurry was also cooked into a smooth flowing porridge as described above. Spices were not added to prevent the masking of the starter culture attributes.

All the porridge samples prepared with the starter culture and inoculum enrichment as described above were assessed by a panel for a laboratory-based consumer acceptability test. The best five starter culture combinations were selected for further work.

The best five starter culture combinations were each used to prepare *Hausa koko* by the traditional process with a little modification for improved safety. Millet grains were de-stoned and winnowed in the laboratory. For each starter culture combination, 200 g was weighed, washed and de-stoned again in water three times. The millet grains were steamed over boiling water in a strainer for 10 min (selected based on microbial analyses outcomes) to reduce their microbial load. It was allowed to cool for 30 min, steeped in 100 ml of water which had been boiled and cooled for 30 min and inoculated with the starter culture combination. It was allowed to ferment for 6 h, sieved to drain away the water, and milled with spices. The spices used were ginger, 10 g, dried red finger hot chile pepper, 1.3 g, Ethiopian/Negro pepper (Twi *hwentia*) 3.0 g, West African black pepper (Twi *esoro wisa*) 1.5g and cloves (Twi *pepre*) 1.0 g. All the spices had been washed thrice with 25 % vinegar solution to reduce the microbial load. The wet milling was done using a lab blender (Akai BD031A-767, Japan) which was cleaned with 70 % alcohol and rinsed with boiling water (100 ºC). The slurry was prepared with 800 ml water which had been boiled for 5 min and cooled for 30 min. The spiced millet slurry was sieved to remove chaff using a fine sieve, covered, and allowed to ferment for another 6 h at room temperature (ca 28 ºC) (without further inoculation).

Each of the fermented slurry was cooked into *Hausa koko* by mixing it with 1.2 litres of boiling water (100 ºC). The mixture was boiled again for two minutes and the samples assessed by a panel for consumer acceptability.

6.2.7.2 Laboratory based sensory evaluation by panel

The three batches of samples that underwent laboratory-based acceptability tests contained (i) starter culture as inoculum enrichment i.e. starter culture in non-sterile millet flour, (ii) Starter culture and sterile millet flour, (iii) starter culture as inoculum enrichment i.e. starter culture in modified traditional *Hausa koko* process. The sensory panel that undertook the acceptability test was made up of twenty untrained panelists (Edima-Nyah et al., 2019; Ubbor & Akobundu, 2009; Okoli & Adeyemi, 1989), who were regular consumers of *Hausa koko*. They were selected from staff of CSIR-Food Research Institute. The same panel members carried out all the three (3) assessment for consumer acceptability. The *Hausa koko* samples were evaluated based on consistency (thickness), colour, taste, aroma and overall acceptability. The different samples were randomly coded using three figures and assessed on a nine-point hedonic scale ranging from 1 meaning dislike extremely to 9 meaning like extremely as shown below:

9 Like Extremely
8 Like Very Much
7 Like Moderately
6 Like Slightly
5 Neither Like nor Dislike
4 Dislike Slightly
3 Dislike Moderately
2 Dislike Very Much
1 Dislike Extremely.

The scores from the scale ratings were subjected to Analysis of Variance (ANOVA) and Duncan test (SPSS version 21.0). Differences in the samples were assessed by Principal Component Analysis (PCA) using XLSTAT 2014.5.03. A significant level of $P \leq 0.05$ was used.

6.3 Results

6.3.1. Starter culture development

6.3.1.1 Antimicrobial interactions between selected isolates

The results of the antimicrobial interactions showed there were no interactions between the LAB isolates nor LAB and yeast isolates (Table 15a and 15b).

Table 15a: Antimicrobial interaction between selected LAB and yeast isolates (in two's) for starter culture

Isolates	**Indicator Strains**					
	L. fermentum LMAN-Sdb	*L. reuteri* LDOD-Sud	*L. pontis* LTAD-12g	*S. cerevisiae* YSUN-Sud	*P. kudriavzevii* YTAD-12j	Control (Media)
L. fermentum LMAN-Sdb	-	-	-	-	-	
L. reuteri LDOD-Sud	-	-	-	-	-	
L. pontis LTAD-12g	-	-	-	-	-	
S. cerevisiae YSUN-Sud	-	-	-	-	-	
P. kudriavzevii YTAD-12j	-	-	-	-	-	
Control (Media)						-

NB: - = No inhibition

Table 15b: Antimicrobial interaction between selected LAB and yeast isolates (in three's) for starter culture

Isolates	Indicator Strains	
	S. cerevisiae YSUN-Sud	*P. kudriavzevii* YTAD-12j
L. reuteri LDOD-Sud + *L. fermentum* LMAN-Sdb	-	-
L. reuteri LDOD-Sud + *L. pontis* LTAD-12g	-	-
L. pontis LTAD-12g + *L. fermentum* LMAN-Sdb	-	-

NB: - = No inhibition

6.3.1.2 pH and microbial changes during starter culture fermentation

The selected LAB and yeast isolates for starter culture development were paired in 15 combinations of two's and three's. The combinations were inoculated into 200 ml sterile millet slurry (10 %) in duplicates whilst a non-inoculated sterile broth was used as a control for comparative purposes. Their pH levels monitored over 12 h period clearly showed that both the double and triple combinations were able to acidify below pH 4 (Figure 18). The starter cultures were able to reduce the pH ranging from 6.35-6.63 at 0 h to 3.39-3.80 at 12 h. The controlled sample did not acidify as much as the starter cultures, starting at 6.76 and ended at 5.72. There were significant differences ($P \leq 0.05$) between the control and all the starter culture-fermented slurries. Some of the double combinations even produced lower pH values than some of the triple ones even though the lowest pH was produced by PFC combination (3.39 ± 0.01). For instance, sample RP at 12 h of fermentation recorded a pH value of 3.48 ± 0.01 which was significantly different ($P \leq 0.05$) from sample RFK of pH value 3.80 ± 0.01.

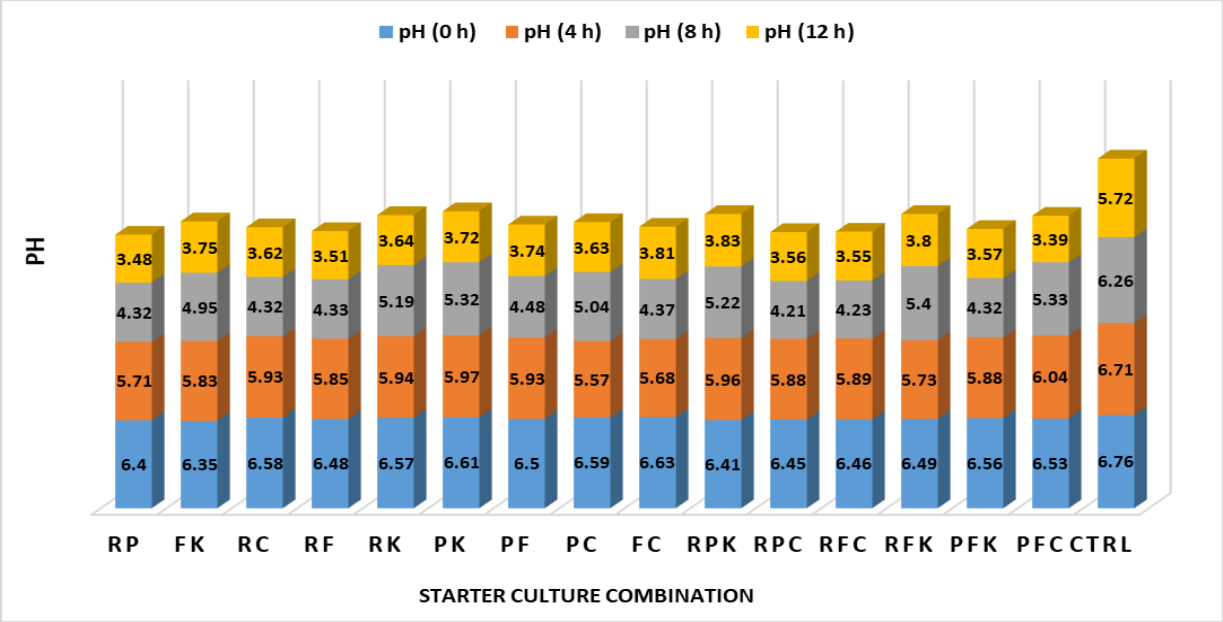


Figure 18: pH changes during 12 hours fermentation of millet slurries using different starter culture combinations

The LAB and yeast population of starter culture fermentation with the different combinations and control for 12 h is presented in Table 16. The population of LAB at the start of fermentation ranged from log 5.25 ± 0.03 - 6.72 ± 0.05 CFU/g and ended at a population range of log 8.54 ± 0.05 - 9.78 ± 0.05 CFU/g. Yeast populations started from log 4.40 ± 0.08 - 5.86 ± 0.03 and ended at log 7.65 ± 0.06 - 8.93 ± 0.02 CFU/g. The combinations that yielded the highest LAB and yeast populations at the end of 12 h fermentation were RC, RF, RP, RFC, PFC and PFK. The control sample recorded the lowest LAB and yeast populations of log 5.62 ± 0.04 and 4.75 ± 0.03 CFU/g respectively by the end of 12 h fermentation. Generally, there were significant differences ($P \leq 0.05$) between the different fermentation time points for both LAB and yeast population for the different starter culture combinations. There were significant differences ($P \leq 0.05$) between the control and all the starter culture combinations.

Table 16: LAB and yeast counts (Log CFU/ml) during starter culture fermentation

Sample	RP	FK	RC	RF	RK	PK	PF	PC
LAB								
0 h	6.45±0.08^{c}	5.97±0.01^{d}	6.71±0.01^{d}	6.72±0.05^{d}	5.25±0.03^{d}	5.80±0.06^{d}	5.78±0.04^{d}	5.42±0.12^{d}
4 h	7.73±0.02^{bc}	6.42±0.06^{c}	7.42±0.06^{c}	7.87±0.01^{c}	5.92±0.01^{c}	6.72±0.05^{c}	6.69±0.08^{c}	5.93±0.01^{c}
8 h	8.44±0.73^{ab}	7.75±0.01^{b}	8.70±0.02^{b}	8.55±0.06^{b}	7.92±0.03^{b}	7.92±0.03^{b}	7.96±0.01^{b}	7.81±0.03^{b}
12 h	9.45±0.01^{a}	8.95±0.04^{a}	9.43±0.02^{a}	9.78±0.02^{a}	8.54±0.02^{a}	8.92±0.01^{a}	8.82±0.01^{a}	8.62±0.01^{a}
Yeast								
0 h	5.73±0.01^{d}	4.60±0.03^{d}	4.61±0.03^{d}	5.86±0.03^{c}	4.96±0.01^{d}	4.40±0.08^{d}	4.65±0.05^{d}	4.46±0.02^{d}
4 h	5.93±0.01^{c}	4.87±0.02^{c}	5.80±0.01^{c}	5.94±0.02^{c}	5.53±0.12^{c}	4.91±0.01^{c}	4.98±0.02^{c}	5.87±0.02^{c}
8 h	7.73±0.02^{b}	6.85±0.10^{b}	7.88±0.02^{b}	7.65±0.05^{b}	6.48±0.04^{b}	6.67±0.03^{b}	6.79±0.08^{b}	6.81±0.03^{b}
12 h	8.30±0.09^{a}	7.95±0.02^{a}	8.93±0.02^{a}	8.70±0.02^{a}	7.74±0.02^{a}	7.95±0.01^{a}	7.65±0.06^{a}	7.76±0.01^{a}

Table 16 continuation: LAB and yeast counts (Log CFU/ml) during starter culture fermentation

Sample	FC	RPK	RPC	RFC	RFK	PFK	PFC	Ctrl
LAB								
0 h	5.70±0.02^{d}	5.71±0.06^{d}	5.61±0.09^{d}	6.62±0.19^{d}	5.77±0.07^{d}	6.68±0.06^{d}	6.60±0.02^{d}	2.28±0.14^{d}
4 h	6.94±0.03^{c}	5.96±0.02^{c}	6.67±0.03^{c}	7.88±0.01^{c}	6.48±0.03^{c}	7.79±0.03^{c}	7.64±0.10^{c}	2.83±0.05^{c}
8 h	7.89±0.02^{b}	7.75±0.05^{b}	7.93±0.01^{b}	8.75±0.03^{b}	7.74±0.04^{b}	8.87±0.03^{b}	8.20±0.08^{b}	4.66±0.09^{b}
12 h	8.54±0.05^{a}	8.93±0.01^{a}	8.81±0.02^{a}	9.78±0.05^{a}	8.95±0.06^{a}	9.46±0.12^{a}	9.75±0.02^{a}	5.62±0.04^{a}
Yeast								
0 h	4.52±0.04^{d}	4.74±0.02^{d}	4.73±0.03^{d}	5.65±0.10^{d}	4.60±0.08^{d}	4.80±0.03^{d}	5.54±0.03^{d}	1.48±0.12^{d}
4 h	4.91±0.02^{c}	4.94±0.02^{c}	4.96±0.01^{c}	5.81±0.03^{c}	4.76±0.11^{c}	5.94±0.04^{c}	5.90±0.02^{c}	2.63±0.03^{c}
8 h	6.65±0.01^{b}	6.81±0.02^{b}	6.56±0.04^{b}	7.72±0.02^{b}	6.89±0.01^{b}	7.57±0.02^{b}	7.57±0.11^{b}	3.85±0.02^{b}
12 h	7.75±0.02^{a}	7.88±0.03^{a}	7.81±0.02^{a}	8.92±0.02^{a}	7.77±0.03^{a}	8.75±0.04^{a}	8.56±0.03^{a}	4.75±0.03^{a}

NB: Means and standard deviations across a column with different letters (superscripts) are significantly different at $P \leq 0.05$. (LAB was analysed separately from yeast)

NB:
RP = *L. reuteri* LDOD-Sud + *L. pontis* LTAD-12g;
RF = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb
RC = *L. reuteri* LDOD-Sud + *S. cerevisiae* YSUN-Sud;
RK = *L. reuteri* LDOD-Sud + *P. kudriavzevii* YTAD-12j;
PK = *L. pontis* LTAD-12g + *P. kudriavzevii* YTAD-12j;
PF = *L. pontis* LTAD-12g + *L. fermentum* LMAN-Sdb;
PC = *L. pontis* LTAD-12g + *S. cerevisiae* YSUN-Sud;
FC = *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;
FK = *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j;
RPC = *L. reuteri* LDOD-Sud + *L. pontis* LTAD-12g + *S. cerevisiae* YSUN-Sud;
RPK = *L. reuteri* LDOD-Sud + *L. pontis* LTAD-12g + *P. kudriavzevii* YTAD-12j;
RFC = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;
RFK = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j;
PFC = *L. pontis* LTAD-12g + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;
PFK = *L. pontis* LTAD-12g + *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j.

6.3.1.3 Effect of starter culture fermentation on aflatoxin level

The use of the various starter cultures in the fermentation of millet slurry for 12 hours resulted in a greater reduction in the content of aflatoxins in the slurry in comparison to the spontaneous fermentation of the slurry as seen in Table 17. With regards to aflatoxin B1 which was present in the slurries at a concentration of 40.48 ± 1.82 µg/kg before fermentation, the spontaneously fermented slurry showed a concentration of 32.26 ± 0.40 µg/kg representing reduction of 20.3 %. Aflatoxin B1 was no longer detected in 6 out of the 15 samples fermented with starter culture. Out of the 9 samples of the slurries fermented with starter culture which showed the presence of aflatoxin B1, the concentrations detected were in the range of 3.26 ± 0.15 to 11.57 ± 0.13 µg/kg representing a reduction of 91.9 % to 71.4 % compared to 20.3 % in the spontaneously fermented sample. Aflatoxin B2 which was present in the millet slurry at a concentration of 1.78 ± 0.03 µg/kg, was no longer detected in the spontaneously fermented sample as well as all the samples fermented with the different combinations of starter culture. Aflatoxins G1 was not detected in the control (0

h) sample and as such was absent in all the 12 h fermented samples. Aflatoxins G2 was not detected in any of the 12 h starter culture fermented samples though it was present at the start of the fermentation in the same concentrations recorded for the control (0 h) since it was the same batch of millet slurry which was used for the spontaneous fermentation as well as the starter culture fermentations. Even though a reduction was recorded in the 12 h fermented control, this was not significant ($P \leq 0.05$). In all, none of the aflatoxins, B1, B2, G1 and G2 were detected in the aflatoxin contaminated millet slurries fermented with RP, PK, PC, FC, FK, RPC or PFC. A comparism of all the fermented samples with the control showed significant differences ($P \leq 0.05$). A comparison of all samples which were detected for AFB1 to the EU regulatory maximum limit of 2 µg/kg (European Commission, 2010) for cereals showed that aside sample RF (12h) whose mean (3.26) was not statistically different ($P \leq 0.05$) from the EU limit, all others with AFB1 detected had levels significantly higher ($P \leq 0.05$) than the regulatory limit. The total maximum limit set for ceareals by the Ghana Standards Authority is however 15 µg/kg (Ghana Standards Authority, 2013) making these results acceptable in Ghana.

Table 17: Effect of 12 h starter culture fermentation on aflatoxin levels in contaminated millet slurries.

Sample Code	AFB1 (µg/kg)	AFB2 (µg/kg)	AFG1 (µg/kg)	AFG2 (µg/kg)
Ctrl (0 h)	40.48 ± 1.82^{a}	1.78 ± 0.03^{a}	0	23.41 ± 2.02^{a}
RP (12 h)	0^{g}	0^{b}	0	0^{b}
RC (12 h)	10.06 ± 0.34^{cd}	0^{b}	0	0^{b}
RF (12 h)	3.26 ± 0.15^{f}	0^{b}	0	0^{b}
RK (12 h)	4.59 ± 0.14^{ef}	0^{b}	0	0^{b}
PK (12 h)	0^{g}	0^{b}	0	0^{b}
PF (12 h)	10.55 ± 0.04^{cd}	0^{b}	0	0^{b}
PC (12 h)	0^{g}	0^{b}	0	0^{b}
FC (12 h)	0^{g}	0^{b}	0	0^{b}
FK (12 h)	0^{g}	0^{b}	0	0^{b}
RPC (12 h)	0^{g}	0^{b}	0	0^{b}
RPK (12 h)	7.60 ± 0.31^{de}	0^{b}	0	0^{b}
RFC (12 h)	3.72 ± 0.10^{f}	0^{b}	0	0^{b}
RFK (12 h)	11.57 ± 0.13^{c}	0^{b}	0	0^{b}
PFK (12 h)	8.40 ± 0.11^{cd}	0^{b}	0	0^{b}
PFC (12 h)	0^{g}	0^{b}	0	0^{b}
Ctrl (12 h)	32.26 ± 0.40^{b}	0^{b}	0	20.68 ± 1.59^{a}
Limit (GSA, 2013)	15			
Limit (EU, 2010)	2			

NB: Means and standard deviations across a column with different letters are significantly different at $P \leq 0.05$

The pH of the aflatoxin contaminated slurries were monitored during fermentation (0, 8, 12 h) and the results are given in Table 18. Whilst the pH of the spontaneous was 5.99, pH of the samples fermented with stater cultures ranged from 3.39 -3.93. There were significant differences ($P \leq 0.05$) among the starter culture fermented slurries as well as between the control (without starter culture) sample and all the starter culture-fermented slurries.

Table 18: pH values of 12 h starter culture fermentation of aflatoxin contaminated millet slurries

Starter Combination	0 h	8 h	12 h
RP	6.36^{b}	5.45^{a}	3.39^{a}
RC	6.48^{f}	5.71^{b}	3.64^{f}
RF	6.34^{a}	4.34^{c}	3.62^{e}
RK	6.48^{f}	5.80^{d}	3.73^{hi}
PK	6.55^{i}	5.74^{e}	3.49^{b}
PF	6.41^{d}	4.06^{f}	3.72^{h}
PC	6.53^{h}	5.59^{i}	3.56^{d}
FC	6.52^{g}	4.35^{j}	3.93^{l}
FK	6.52^{g}	4.95^{k}	3.75^{j}
RPC	6.34^{a}	5.17^{m}	3.56^{d}
RPK	6.37^{bc}	5.22^{n}	3.54^{c}
RFC	6.38^{c}	4.50^{p}	3.92^{l}
RFK	6.42^{d}	4.61^{q}	3.90^{k}
PFK	6.43^{e}	4.02^{r}	3.74^{i}
PFC	6.37^{bc}	4.37^{s}	3.69^{g}
Control	6.82^{j}	6.45^{t}	5.99^{m}

Note: Means across a column with different letters are significantly different at $P \leq 0.05$

NB:
RP = *L. reuteri* LDOD-Sud + *L. pontis* LTAD-12g;
RF = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb;
RC = *L. reuteri* LDOD-Sud + *S. cerevisiae* YSUN-Sud;
RK = *L. reuteri* LDOD-Sud + *P. kudriavzevii* YTAD-12j;
PK = *L. pontis* LTAD-12g + *P. kudriavzevii* YTAD-12j;
PF = *L. pontis* LTAD-12g + *L. fermentum* LMAN-Sdb;
PC = *L. pontis* LTAD-12g + *S. cerevisiae* YSUN-Sud;
FC = *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;
FK = *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j;
RPC = *L. reuteri* LDOD-Sud + *L. pontis* LTAD-12g + *S. cerevisiae* YSUN-Sud;
RPK = *L. reuteri* LDOD-Sud + *L. pontis* LTAD-12g + *P. kudriavzevii* YTAD-12j;
RFC = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;
RFK = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j;
PFC = *L. pontis* LTAD-12g + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;
PFK = *L. pontis* LTAD-12g + *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j.

6.3.2 Sensory evaluation

6.3.2.1 Sensory evaluation of millet porridge produced by inoculum enrichment

The first sensory evaluations was an assessment of the degree of likeness for organoleptic properties of millet porridge that had been produced from millet slurry fermented with the starter cultures as inoculum enrichment but did not contain any spices as *Hausa koko* would. Thus, the slurry fermented was not sterile but contained all the natural microbiota of the millet in addition to the starter culture which was added. The mean scores of ratings by the 20-member sensory panel of the millet porridge produced by various starter cultures as inoculum enrichment on a 9 point hedonic scale is shown in Table 19. In all there were very little differences in the mean scores for the various sensory attributes, aroma, colour, consistency and taste as well as overall acceptability among the products. In fact, there was no significant differences ($P \leq 0.05$) between the taste of all the 16 samples and also in the overall acceptability of all the samples. The scores for aroma ranged between 5.65 and 7.25 (liked slightly to liked very much), colour between 6.00 and 7.00 (liked moderately to liked very much), consistency 5.40 and 6.75 (liked slightly to liked moderately), taste 5.35 and 6.70 (liked slightly to liked moderately) and overall acceptability 5.55 and 6.85 (liked slightly to liked moderately). RFC, the porridge fermented by inoculum enrichment with *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud, had the highest score for aroma, taste and overall acceptability. RK, the porridge fermented by inoculum enrichment with *L. reuteri* LDOD-Sud + *P. kudriavzevii* YTAD-12j, had the highest score for colour and PFK, the porridge fermented by inoculum enrichment with *L. pontis* LTAD-12g + *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j, had the highest score for consistency.

A Principal Component Analysis (PCA) using XLSTAT 2014.5.03 of the results showed distinct differences in the samples in general. The first two principal components accounted for more than 80 % of the variability in the sensory data. Acceptability of samples were heavily dependent factors related to oral or nasal senses, rather than physical properties such as colour and consistency. The plot shows that colour and consistency were more closely related in the inoculum enriched millet porridge (Figure 20).

Table 19: Sensory scores for millet porridge produced with different starter cultures as inoculum enrichment

Samples	Aroma	Colour	Consistency	Taste	Overall Acceptability
PFC	6.90 ± 1.33^{ab}	6.45 ± 1.00^{a}	6.05 ± 1.72^{ab}	5.95 ± 1.82^{a}	6.05 ± 1.85^{a}
PFK	6.30 ± 1.60^{ab}	6.35 ± 1.16^{a}	6.95 ± 1.19^{a}	6.20 ± 1.94^{a}	6.20 ± 1.47^{a}
RPC	6.35 ± 1.23^{ab}	6.10 ± 1.41^{a}	5.90 ± 1.37^{ab}	6.10 ± 1.65^{a}	6.20 ± 1.67^{a}
RP	6.30 ± 1.03^{ab}	6.55 ± 0.95^{a}	5.75 ± 1.37^{ab}	5.35 ± 1.76^{a}	5.95 ± 1.67^{a}
PC	5.75 ± 1.52^{ab}	6.15 ± 1.31^{a}	5.70 ± 1.53^{ab}	6.25 ± 1.55^{a}	6.10 ± 1.52^{a}
RC	6.50 ± 1.40^{ab}	6.30 ± 0.98^{a}	6.10 ± 1.41^{ab}	6.10 ± 1.80^{a}	6.10 ± 1.59^{a}
RFC	7.25 ± 0.91^{a}	6.55 ± 1.00^{a}	6.95 ± 1.23^{a}	6.70 ± 1.69^{a}	6.85 ± 1.57^{a}
FK	6.35 ± 1.53^{ab}	6.25 ± 1.29^{a}	5.40 ± 1.67^{b}	6.15 ± 1.39^{a}	6.05 ± 1.47^{a}
PK	6.50 ± 1.05^{ab}	6.55 ± 0.89^{a}	6.15 ± 1.39^{ab}	6.00 ± 1.56^{a}	6.25 ± 1.52^{a}
FC	6.70 ± 1.13^{ab}	6.75 ± 1.23^{a}	6.90 ± 1.83^{a}	5.85 ± 1.76^{a}	6.40 ± 1.78^{a}
RF	6.85 ± 1.08^{ab}	6.75 ± 0.85^{a}	6.35 ± 0.99^{ab}	6.15 ± 1.46^{a}	6.50 ± 1.92^{a}
RFK	6.70 ± 1.49^{ab}	6.95 ± 1.19^{a}	6.85 ± 1.42^{ab}	6.25 ± 2.10^{a}	6.75 ± 1.65^{a}
PF	6.15 ± 1.50^{ab}	5.95 ± 1.40^{a}	5.60 ± 1.23^{ab}	5.60 ± 1.60^{a}	5.75 ± 1.52^{a}
RPK	5.65 ± 1.90^{b}	6.00 ± 1.41^{a}	6.05 ± 1.64^{ab}	5.65 ± 1.53^{a}	5.55 ± 1.96^{a}
RK	6.35 ± 1.55^{ab}	7.00 ± 1.03^{a}	6.55 ± 0.95^{ab}	6.35 ± 1.31^{a}	6.45 ± 1.28^{a}
Control	6.30 ± 1.78^{ab}	6.75 ± 1.21^{a}	6.50 ± 1.64^{ab}	6.00 ± 1.97^{a}	6.15 ± 1.96^{a}

Note: Means across a column with different letters are significantly different at $P \leq 0.05$

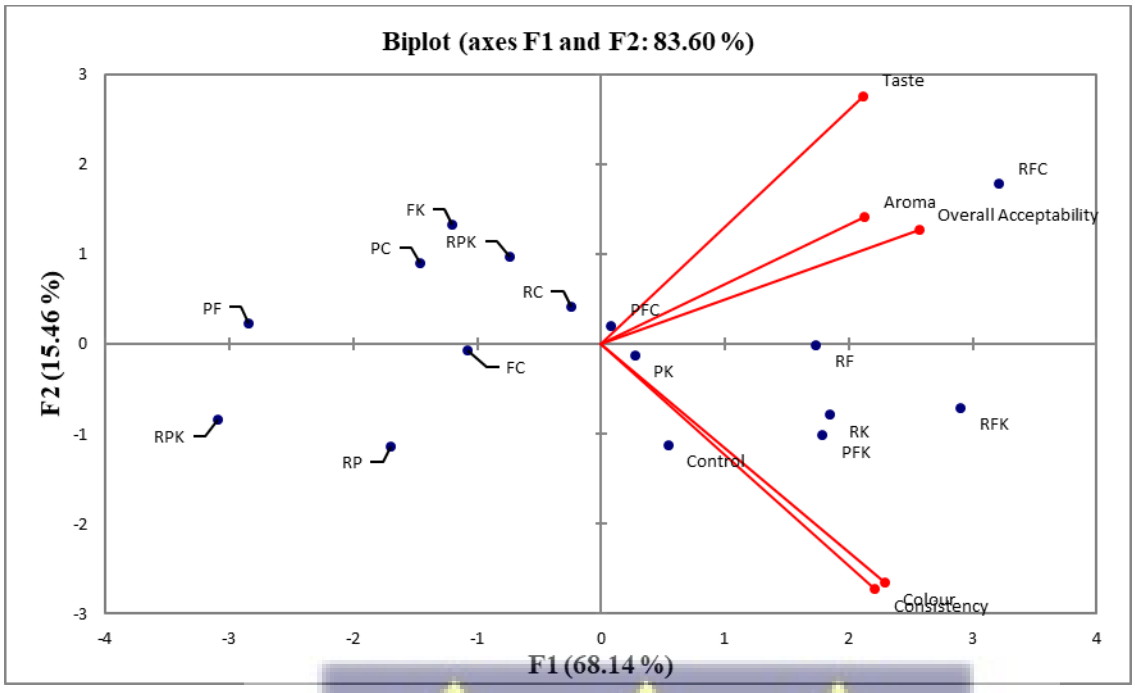


Figure 19. PCA biplot based on the sensory data on *Hausa koko* prepared from non-irradiated fermented slurries inoculated with different culture combinations

NB:

RP = *L. reuteri* LDOD-Sud + *L. pontis* LTAD-12g;

RF = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb

RC = *L. reuteri* LDOD-Sud + *S. cerevisiae* YSUN-Sud;

RK = *L. reuteri* LDOD-Sud + *P. kudriavzevii* YTAD-12j;

PK = *L. pontis* LTAD-12g + *P. kudriavzevii* YTAD-12j;

PF = *L. pontis* LTAD-12g + *L. fermentum* LMAN-Sdb;

PC = *L. pontis* LTAD-12g + *S. cerevisiae* YSUN-Sud;

FC = *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;

FK = *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j;

RPC = *L. reuteri* LDOD-Sud + *L. pontis* LTAD-12g + *S. cerevisiae* YSUN-Sud;

RPK = *L. reuteri* LDOD-Sud + *L. pontis* LTAD-12g + *P. kudriavzevii* YTAD-12j;

RFC = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;

RFK = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j;

PFC = *L. pontis* LTAD-12g + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;

PFK = *L. pontis* LTAD-12g + *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j.

6.3.2.2 Sensory evaluation of sterile millet porridge produced by starter cultures

The second sensory evaluation was an assessment of millet porridge which had been produced from millet slurry fermented by only the starter cultures because the millet grains had been sterilized by irradiation before processing. The mean score of ratings by the 20-member sensory panel of the sterile millet porridge produced by various starter cultures on a 9-point hedonic scale is shown in Table 20. There were no significant differences ($P \leq 0.05$) in the mean scores for the various attributes evaluated except for aroma. The mean scores for aroma ranged from 5.5-7.05 (neither like nor dislike to like moderately), colour ranged from 6.0-6.95 (like slightly), consistency ranged from 5.4-6.85 (neither like nor dislike to like slightly), taste ranged from 5.5-6.75 (neither like nor dislike to like slightly) whilst overall acceptability ranged from 5.45-7.05 (neither like nor dislike to like moderately). sRF the sterile millet porridge fermented with *L. reuteri LDOD-Sud* + *L. fermentum* LMAN-Sdb recorded the highest mean score in all the attributes evaluated. These were 6.95 in terms of colour, 6.85 in terms of consistency and 7.05 in terms of aroma and overall acceptability. sRFC and sRFK, the sterile millet porridges fermented with *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud, and *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j respectively were scored second and third highest in overall acceptability. The taste of the other two sterile porridges most preferred were those fermented with *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud (6.70) and *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud (6.45). The use of sterile millet for the preparation of porridges were not liked much.

The PCA biplot showed that most of the sterile (irradiated) fermented millet porridges appeared to be closely related with very few distinct one's in general. Consistency, taste and colour were

closely related, and acceptability of these samples were influenced by aroma as shown in the PCA plot (Figure 20).

Table 20: Sensory scores for sterile millet porridge produced with different starter cultures.

Samples	Aroma	Colour	Consistency	Taste	Overall Acceptability
sPFC	6.75 ± 0.42^{ab}	6.25 ± 1.34^{a}	6.00 ± 1.60^{a}	5.95 ± 1.53^{a}	6.00 ± 1.90^{a}
sPFK	6.40 ± 1.39^{ab}	6.45 ± 1.08^{a}	6.05 ± 1.31^{a}	6.25 ± 2.10^{a}	6.25 ± 1.05^{a}
sRPC	6.35 ± 1.14^{ab}	6.15 ± 1.11^{a}	5.85 ± 1.24^{a}	6.25 ± 1.55^{a}	6.20 ± 1.43^{a}
sRP	6.20 ± 1.40^{ab}	6.45 ± 0.70^{a}	5.65 ± 1.44^{a}	5.70 ± 1.47^{a}	5.90 ± 1.81^{a}
sPC	5.60 ± 1.22^{b}	6.15 ± 1.66^{a}	5.85 ± 1.33^{a}	6.05 ± 1.93^{a}	6.15 ± 1.92^{a}
sRC	6.60 ± 1.30^{ab}	6.40 ± 0.88^{a}	6.10 ± 1.90^{a}	6.15 ± 1.63^{a}	6.10 ± 1.78^{a}
sRFC	6.75 ± 0.76^{ab}	6.85 ± 0.90^{a}	6.40 ± 1.44^{a}	6.70 ± 0.99^{a}	6.85 ± 0.76^{a}
sFK	6.20 ± 1.74^{ab}	6.15 ± 1.32^{a}	5.50 ± 1.91^{a}	6.10 ± 1.98^{a}	6.10 ± 0.64^{a}
sPK	6.50 ± 0.23^{ab}	6.30 ± 0.45^{a}	6.15 ± 1.02^{a}	6.10 ± 1.32^{a}	6.15 ± 1.49^{a}
sFC	6.60 ± 1.28^{ab}	6.35 ± 1.44^{a}	6.40 ± 0.57^{a}	6.45 ± 1.84^{a}	6.30 ± 1.82^{a}
sRF	7.05 ± 1.49^{a}	6.95 ± 1.04^{a}	6.85 ± 0.21^{a}	6.75 ± 0.68^{a}	7.05 ± 1.02^{a}
sRFK	6.50 ± 1.60^{ab}	6.75 ± 1.34^{a}	6.75 ± 1.21^{a}	6.25 ± 1.74^{a}	6.55 ± 0.63^{a}
sPF	6.05 ± 1.71^{ab}	6.00 ± 1.80^{a}	5.40 ± 1.01^{a}	5.50 ± 1.50^{a}	5.90 ± 1.70^{a}
sRPK	5.60 ± 0.73^{b}	6.10 ± 0.99^{a}	6.15 ± 1.70^{a}	5.95 ± 0.84^{a}	5.45 ± 1.60^{a}
sRK	6.70 ± 1.41^{ab}	6.80 ± 1.03^{a}	6.60 ± 1.05^{a}	6.30 ± 0.22^{a}	6.50 ± 1.32^{a}
Control	6.40 ± 1.34^{ab}	6.60 ± 0.56^{a}	6.40 ± 1.80^{a}	6.15 ± 0.74^{a}	6.30 ± 1.27^{a}

Note: Means across a column with different letters are significantly different at $P \leq 0.05$
s = irradiated millet

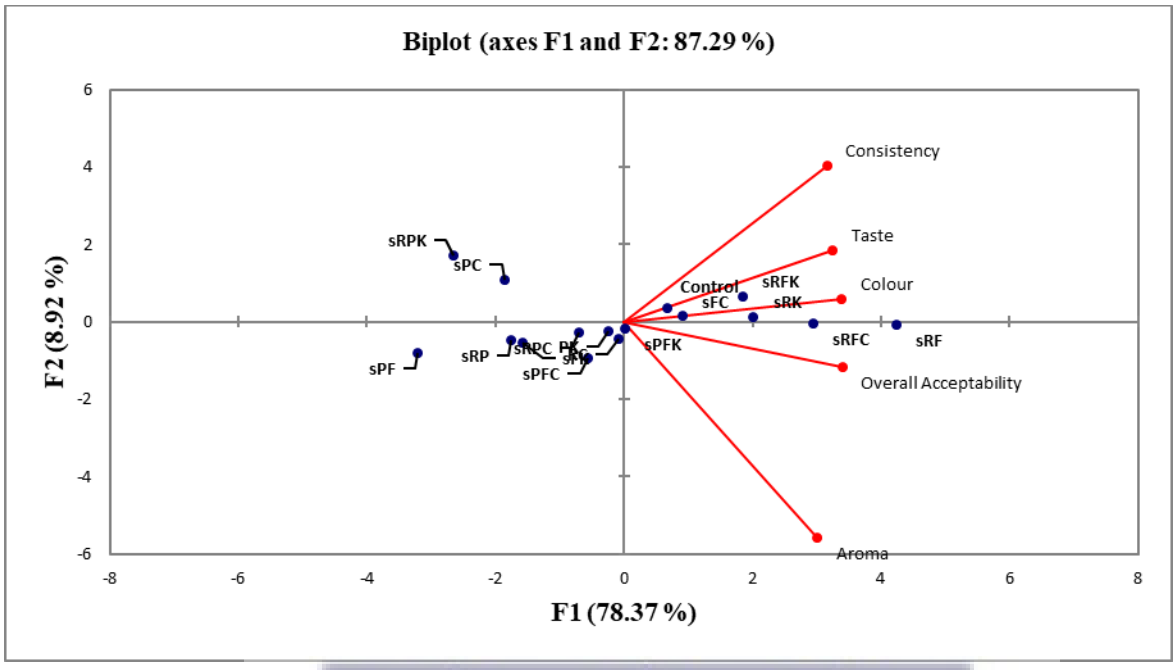


Figure 20: PCA biplot based on the sensory data on *Hausa koko* prepared from irradiated fermented slurries inoculated with different culture combinations

NB:

RP = *L. reuteri* LDOD-Sud + *L. pontis* LTAD-12g;

RF = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb

RC = *L. reuteri* LDOD-Sud + *S. cerevisiae* YSUN-Sud;

RK = *L. reuteri* LDOD-Sud + *P. kudriavzevii* YTAD-12j;

PK = *L. pontis* LTAD-12g + *P. kudriavzevii* YTAD-12j;

PF = *L. pontis* LTAD-12g + *L. fermentum* LMAN-Sdb;

PC = *L. pontis* LTAD-12g + *S. cerevisiae* YSUN-Sud;

FC = *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;

FK = *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j;

RPC = *L. reuteri* LDOD-Sud + *L. pontis* LTAD-12g + *S. cerevisiae* YSUN-Sud;

RPK = *L. reuteri* LDOD-Sud + *L. pontis* LTAD-12g + *P. kudriavzevii* YTAD-12j;

RFC = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;

RFK = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j;

PFC = *L. pontis* LTAD-12g + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;

PFK = *L. pontis* LTAD-12g + *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j.

A comparison between the first and second batch showed that these samples that did not contain spices were not liked very much. The overall acceptability scores ranged from 5.55 (liked slightly) to 6.85 (liked moderately) was obtained for the inoculum enriched samples whilst it ranged from 5.45 (neither liked nor disliked) to 7.05 (liked moderately).

6.3.2.3 Sensory evaluation of *Hausa koko* produced using different starter cultures

In the third sensory evaluation for consumer acceptability, the outcomes from the two previous evaluations were used in selecting five (RFC, RFK, RK, RF and FC) out of the 15 different combinations for the final selection process prepared following the traditional *Hausa koko* process with added spices. The pH of these samples after preparation were 3.54, 3.3, 3.48, 3.56, 3.57 and 5.68 for RFC, RFK, RK, RF, FC and control respectively. Generally, these samples were rated higher than the unspiced millet porridges evaluated previously and presented in Table 21. Sample RFC which was fermented with *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud which had been rated highly in the millet porridge assessments was rated highest except for colour in all categories evaluated by the 20-member panel. It had a mean score of 8.43 (like very much) for overall acceptability with the other attributes which were significantly different ($P \leq 0.05$) from the other samples and control evaluated.

The visualization of these samples is shown in Figure 21. The plot indicates dissimilarities between the best five samples. Sample RKF was closely related to the control as there was little dissimilarity between them in terms of overall acceptability whilst RF and FC were also closely related to each other in terms of their aroma and consistency. Sample RFC which was highly rated was the most dissimilar to the control.

Table 21: Sensory results of *Hausa koko* prepared using the five preferred starter culture combinations following the traditional processing method B.

Samples	Aroma	Colour	Consistency	Taste	Overall Acceptability
Control	6.91 ± 0.63^{abc}	6.86 ± 1.56^{abc}	7.10 ± 0.63^{abc}	6.86 ± 1.11^{abc}	7.05 ± 0.87^{bc}
RFC	7.94 ± 0.51^{a}	7.31 ± 0.68^{b}	7.78 ± 0.48^{a}	7.96 ± 0.66^{a}	8.43 ± 0.77^{a}
RK	6.33 ± 1.56^{c}	6.19 ± 1.47^{c}	6.37 ± 1.40^{c}	6.14 ± 1.53^{c}	6.48 ± 1.03^{c}
FC	7.52 ± 0.51^{ab}	7.46 ± 0.81^{ab}	7.19 ± 0.96^{abc}	7.29 ± 0.85^{ab}	7.67 ± 0.73^{ab}
RF	7.29 ± 0.72^{ab}	7.10 ± 1.10^{abc}	7.38 ± 0.75^{ab}	7.00 ± 1.23^{abc}	7.48 ± 0.87^{b}
RFK	6.66 ± 1.56^{bc}	6.74 ± 1.19^{bc}	6.55 ± 1.36^{bc}	6.81 ± 1.62^{bc}	6.95 ± 1.36^{bc}

Note: Means across a column with different letters are significantly different at $P \leq 0.05$

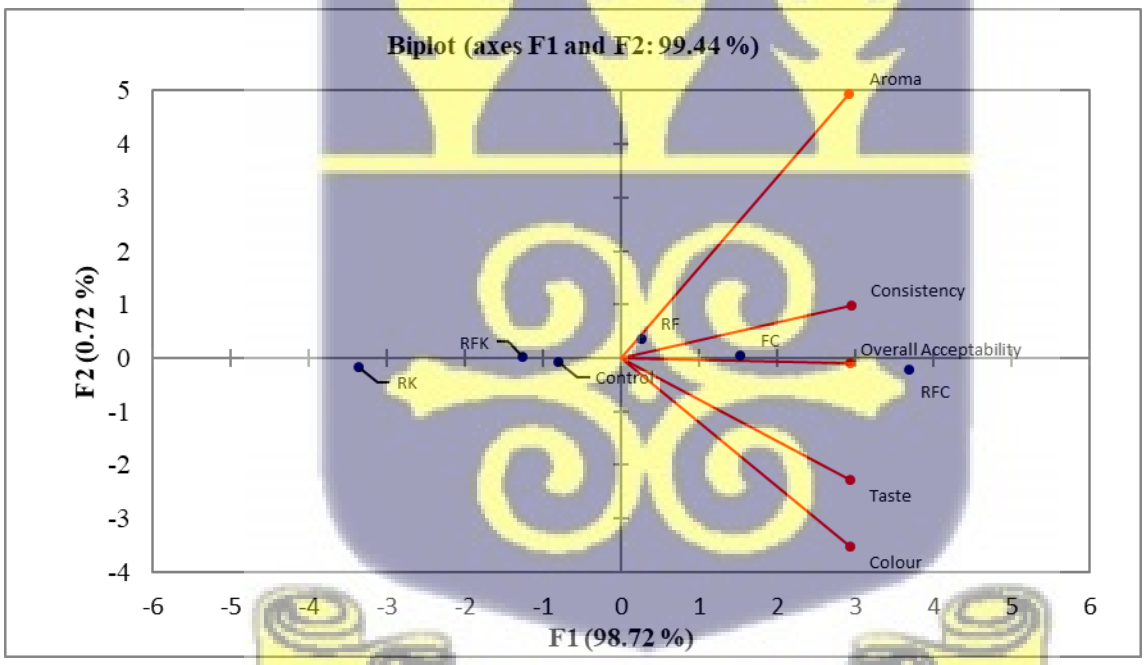


Figure 21: PCA biplot based on the sensory data from *Hausa koko* prepared using the five preferred starter culture combinations following the traditional processing method B.

NB:

RF = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb

RK = *L. reuteri* LDOD-Sud + *P. kudriavzevii* YTAD-12j;

FC = *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;

RFC = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud;

RFK = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *P. kudriavzevii* YTAD-12j;

6.4 Discussion

The isolates of lactic acid bacteria which were selected for development of a starter culture for *Hausa koko* were cultures which had already demonstrated the potential for probiotic activity and other relevant technological properties. These technological and probiotic properties were determined by their abilities to produce exopolysaccharides (EPS), amylase, antimicrobial activity, fast acidification rate, tolerance to bile and low pH. The yeast isolates used had also demonstrated important technological and probiotic properties such as tolerance to high temperatures, low pH and bile. These isolates complemented each other. All the LAB and yeasts cultures had been isolated from spontaneously fermenting millet slurry during *Hausa koko* production. The LAB were strains of *Limosilactobacillus reuteri*, *Limosilactobacillus pontis*, *Limosilactobacillus fermentum*, and the yeasts *Saccharomyces cerevisiae*, and *Pichia kudriavzevii*. The LAB had been identified by sequencing of the whole genome using the Illumina HiSeq4000 platform and the yeasts by sequencing of the 28S ribosomal RNA using Sanger technique.

Nearly all the LAB and yeasts in combinations of twos and threes demonstrated good growth when inoculated into millet slurry, either as pure cultures or as inoculum enrichment. Most of them quickly started and accelerated the fermentation process with high populations of LAB and yeast within 12 h. The LAB and yeast populations at the end of 12 h fermentation ranged between log 8.54 - 9.78 CFU/g for LAB and log 7.65 - 8.93 CFU/g for yeasts respectively. All the starter culture combinations of LAB and yeasts were able to rapidly acidify millet slurry within 12 hours. The pH values during the 12-hour period dropped from 6.34 - 6.63 to 3.30 - 3.83. According to De Melo Pereira et al., (2018), decrease in pH is one of the most important factors that microbial strains to be used as starter cultures must exhibit. The acidification of the millet slurry by the LAB was the result of the production of lactic acid and acetic acids by the hetero-fermentative LAB

(*Limosilactobacillus fermentum* LMAN-Sdb, *Limosilactobacillus reuteri* LDOD-Sud, and *Limosilactobacillus pontis* LTAD-12g). Production of the acids resulted in the lowering of pH during fermentation to decrease the pH of the slurries. It was essential to achieve such viable cell populations of LAB and yeast in potential probiotic strains for starter culture consideration and usage in order to attain such desired pH reductions. High viable cell populations with good acidification rates is an important growth limiting attribute by the isolates for foodborne pathogen inhibition (Di Stefano et al., 2017). This trend of high LAB and yeast growth marked with fast acidification rate during cereal fermentation processes has been reported (Houngbédji et al., 2018) and extensively discussed earlier (Chapter 5). Irradiation at 5 kGy dose accounted for the low LAB and yeast counts observed in the control sample fermentation. Irradiation dose range of 5-10 kGy in dry ingredients like the millet flour is reported to reduce the viable microbial counts (European Food Safety Authority, 2011; Wilkinson & Gould, 1996).

Aflatoxin contamination of some food crops such as groundnuts and also cereals such as maize, millet and others is a problem in Ghana. Ability of microbial isolates, in this case LAB and yeast, to reduce the levels of aflatoxins during the fermentation of *Hausa koko* was therefore an important factor assessed during the development of the starter culture. Reduction or total elimination in the levels of aflatoxins G2, B1 and B2 was achieve by the different starter culture combinations within 12 h fermentation in 10 % millet slurries. These toxins result in foodborne outbreaks upon consumption, but the use of starter cultures may effectively reduce or totally prevent the toxins in fermented foods (Sivamaruthi et al., 2019). This observation has been supported by the results obtained in the present study. The impact of the different starter culture combinations on the aflatoxins in the millet slurries were diverse with regards to the extent of reduction or even total inhibition and may be attributed to different reasons. An important reason for the reduction in the

concentration of aflatoxins may be the decrease in pH which ranged between 3.39-3.93 at the end of 12 hours of fermentation. The reduction in pH was considered as an important factor in the enhanced reduction of aflatoxins in the millet slurries during fermentation. This is because pH condition is reported to significantly control the production of antifungal metabolites by LAB (Batish et al., 1997). However, some of the starter culture combinations recorded very low pH values yet still showed the presence of aflatoxin B1 even though reduced. This may also confirm other contrary reports which also suggest a rise in aflatoxin levels in lower pHs as the effect of pH has been associated with other factors such as incubation period, the strains available, competing microbes and temperature (Gourama & Bullerman, 1995). The presence of a lactone ring has also been reported in aflatoxin molecules which re-forms hydrolysed molecules with the closing of the ring in lower pHs or in acidic environment. The reduction in pH levels therefore results in some increment in aflatoxin levels (Kpodo et al., 1996; Price & Jorgensen, 1985).

The interactions between the microbial populations involved during the fermentation may have also played a key role in determining such outcomes influenced by other metabolites, accumulation of by-products as well as their activities and growth kinetics. These thus depicted their level of inhibition (Kovárová-Kovar & Egli, 1998). Its been reported that establishment of unfavourable conditions also diminishes the growth rate of undesirable microorganisms (Bassi et al., 2015) including species of the genera *Aspergillus*, *Fusarium* and *Penicillium*, the mycotoxigenic producing moulds (Dalié et al., 2010). The inhibiting compounds produced by LAB mostly of the genera *Lactobacillus*, *Lactococcus* and sometimes *Pediococcus* and *Leuconostoc* during the fermentation interacts with the mycotoxins resulting in their prevention or decrease (Dalié et al., 2010; Gerez et al., 2009). Most probiotic organisms may remove aflatoxins in cereals by adsorption (Wu et al., 2009; Shetty & Jespersen, 2006). The antifungal ability of LAB during

fermentation depends on the LAB strain available and the fungal species involved in the fermentation matrix (Gerez et al., 2009). For instance, lactic, acetic, propionic and phenyl lactic acids were some antifungal compounds produced by 95 different LAB including strains of *L. fermentum* and *L. reuteri* against moulds (*A. niger, Penicillium* spp, and *F. graminearum*) in breads. Some of these antifungal strains were used together with *S. cerevisiae*. Based on their outcome, Gerez et al., (2009) suggested that LAB may be used in the inhibition of moulds to prevent spoilage on bread. Other strains of *S. cerevisiae* have also been reported to reduce levels of aflatoxin contaminations in fermented foods (Gonçalves et al., 2015; Shetty et al., 2007).

The differences observed in the level of reduction by the different combinations may also be attributed to the level of stability of the complexes or bond formed between the LAB-yeast and the mycotoxins present as the binding strength depends on the individual strains and other environmental conditions. Some LAB also have the ability to trap some mycotoxins (Dalié et al., 2010).

Similarly, the reduction or eradication in levels of aflatoxins in other cereal fermented foods have been reported (Ahlberg et al., 2015). Isolates including *L. fermentum* from indigenous fermented cereal gruels were reported to inhibit aflatoxin B1and G producing *Aspergillus* spp (Onilude et al., 2005). According to Ogunbanwo et al., (2005), aflatoxins B and G were inhibited by *L. plantarum* K1 when used as starter culture during cereal fermentation for the preparation of *ogi*. Muñoz et al., (2010) also reported the growth inhibition properties of *L. fermentum, L. rhamnosus* and *S. cerevisiae* against mycotoxin producing *Aspergillus* strain.

Despite how well a starter culture performs in terms of technological and probiotic potentials, its acceptability by consumers is very important. For that reason, all the combinations were used for *Hausa koko* production and acceptability evaluated. They were prepared using both irradiated and

non-irradiated millet slurries without spices to prevent the masking of the starter culture attributes by the spices. It was however observed that even though these samples without spices were not liked very much, the panel was still able to identify almost the same preferred combinations (RFC, RFK, RK, RF and FC) in both cases. These preferred combinations, which were pre-selected and used for the fermentation of millet for *Hausa koko* production following the traditional process with spices added and some process modification were evaluated again for acceptability. They were judged on their aroma, colour, consistency, taste and overall acceptability by the 20-member panelist generally showed a good acceptability of the samples. However, the most preferred starter culture fermented *Hausa koko* was that fermented by *Limosilactobacillus reuteri* LDOD-Sud (**R**) + *Limosilactobacillus fermentum* LMAN-Sdb (**F**) + *Saccharomyces cerevisiae YSUN-Sud* (**C**) combination. This was significantly different ($P \leq 0.05$) from the others and control. *Hausa koko* samples fermented with *Limosilactobacillus fermentum* LMAN-Sdb (**F**) + *Saccharomyces cerevisiae YSUN-Sud* (**C**) combination and *Limosilactobacillus reuteri* LDOD-Sud (**R**) + *Limosilactobacillus fermentum* LMAN-Sdb (**F**) combinations were the second and third most preferred. These outcomes suggest preferences based on familiarity and the unique sensory characteristics of *L. fermentum, S. cerevisiae* and to some extent *L. reuteri* impacts from other known Ghanaian cereal fermented foods. *L. fermentum* and *S. cerevisiae*, are associated with most cereal fermented foods in Ghana like *kenkey, koko, fura* and others (Annan et al., 2015; Owusu-Kwarteng et al., 2012; 2010; Ackaah-Gyasi, 2010). The outcome also suggests that *Hausa koko* production following the traditional process with addition of spices and some process modification may be the ideal step for inoculation of starter cultures in *Hausa koko* fermentation with good characteristics. The overall acceptability may also be attributed to the pH of the samples as RFC, RF and FC were around pH values comparable with those from commercial processors i.e. 3.54, 3.56 and 3.57 respectively. On the other hand, the samples fermented with cultures containing *P.*

kudriavzevii (RK and RFK) were the most acidic (3.48 and 3.3 respectively) and least preferred. Although RFC was the most acceptable, RF and FC can all be considered ideal for usage as starter cultures in millet fermentation for *Hausa koko* production and for millet fermentation in general. Additionally, there was reduction (RF, 3.26 µg/kg; RFC, 3.72 µg/kg) or eradication (FC, 0 µg/kg) in levels of aflatoxin B1.

6.5 Conclusion

The potential probiotic strains of LAB isolates *L. reuteri* LDOD-Sud (R), *L. pontis* LTAD-12g (P), *L. fermentum* LMAN-Sdb (F) and yeast *S. cerevisiae* YSUN-Sud (S) and *P. kudriavzevii* YTAD-12j (K) were combined in fifteen different combinations of twos and threes for this study. The different combinations demonstrated good acidification rates, high viable cell populations with aflatoxin inhibiting properties within 12 h fermentation. Preferred combinations, which were pre-selected from the millet porridge produced by inoculum enrichment and sterile millet porridge produced by starter cultures both without spices, were then used for the fermentation of millet for *Hausa koko* containing spices following a modified traditional process. The modified traditional process with spices is ideal for inoculation of starter cultures as sample RFC out of the fifteen different combinations gave good sensorial attributes and characteristics. RFC was the most preferred in terms of their acceptability evaluation. It was rated highest in terms of taste, aroma, consistency and overall acceptability except for colour by the 20 member panelists. RFC potential probiotic starter culture may therefore be considered for control fermentation and as inoculum enrichment in indigenous populations. The use of such a starter culture can help in accelerating the fermentation process during millet fermentation in general and *Hausa koko* production with assured preservative properties and acceptability.

CHAPTER SEVEN

7.0 Pilot and semi-industrial scale testing of the use of starter cultures in *Hausa koko* production

7.1 Introduction

According to Holzapfel (2002) and Nout (1992) fermented food processors both at household and small-scale levels who operate under uncontrolled conditions, and in the majority mostly in developing countries, may not be able to apply sophisticated starter culture technologies to fermentation processes. They may lack the technical expertise on the application and monitoring required for controlled fermentation. It is understandable that this is not a common knowledge so they need to be trained to acquire that skill if only such a facility is available which is generally not. Fortunately, in Ghana and several other African countries, many of the indigenous foods are now produced by Small and Medium Scale Enterprises (SME) as convenience foods. Their formal operations at the SME level using mechanized and semi-mechanized operations present an opportunity for the introduction of starter cultures into the production of indigenous African fermented foods.

The challenge of using starter cultures for large scale producers is the equipment required, constant supply of electricity, process control, optimization of conditions including temperature, time, pH, substrate pre-treatment, inoculum-substrate ratio, and standardization of the end-product quality without losing their preferred characteristics (NRC, 1992). Controlled fermentation of cereals using starter cultures as bio preservative agents is common in developed countries (Russo et al., 2017; Axel et al., 2015). Even though a lot of laboratory-controlled fermentations have been

carried out in Africa using lactic acid bacteria (LAB) and yeast, information on fermentations actually carried with these organisms on industrial scale in Africa is scarce (Soro-Yao et al., 2014). In Ghana, controlled fermentation was carried out using starter cultures comprising of six strains of *Limosilactobacillus fermentum* and *Saccharomyces cerevisiae* obtained from traditional maize fermentation to produce *kenkey* (Halm et al., 1996). Out of these, *L. fermentum* (7-11 A) was used as a starter culture to investigate its usage to produce *kenkey* on semi-industrial scale. The starter culture was able to reduce the fermentation time from 48 to 24 h, however routine use of the starter culture by the entrepreneur could not be sustained. There are no other reports of the use of starter culture on a pilot or SME scale in Ghana.

This study was to establish a basis for the possible semi-industrial production of *Hausa koko* flour using starter culture for both the local and international markets. There are presently local SMEs who are engaged in the production of *Hausa koko* flour for these markets but none of these SMEs use starter cultures. The export market for the product is limited and patronized almost exclusively by Ghanaians in the diaspora. Through the use of starter culture, such companies can routinely produce *Hausa koko* flour of consistent sensory quality and also be assured of a safe product as a result of the use of protective probiotic culture. The starter culture which has been developed in the present work for the production of *Hausa koko* and millet fermentation in general is a combination of *Limosilactobacillus reuteri* LDOD-Sud (**R**), *Limosilactobacillus fermentum* LMAN-Sdb (**F**) and the yeast *Saccharomyces cerevisiae* YSUN-Sud (**C**) referred to simply as RFC *Hausa koko* Starter Culture (**RFCH**).

According to Ogunremi et al., (2017) the sophistication of actual food ecosystems is different from *in-vitro* food systems and so it is essential to test the actual expression of the desired characteristics of a potential starter culture *in-situ*. In line with this and for the eventual transfer of the RFCH

Starter Culture technology to semi-industrial scale in future, pilot scale trial was carried out at CSIR-Food Research Institute and an upscaling trial at an SME, Selasie Foods and Groceries Limited. They are engaged in the production of various indigenous Ghanaian foods as convenience foods, for the local and foreign market on a limited scale. Selasie Foods and Groceries Limited produces *Hausa koko* flour and had expressed the desire to use a suitable starter culture which was developed in the present study. This work was therefore carried out to determine the performance of the developed *Hausa koko* starter culture (RFCH) in the fermentation of pearl millet by upscaling the laboratory operations to a pilot scale to assess quality and safety parameters and the possibility of using it at a semi-industrial scale for the production of *Hausa koko* flour.

7. 2 Materials and Methods

7.2.1 Pilot scale fermentation of millet slurry using Starter Culture RFCH

Early millet variety, *Waapp-naara*, obtained from CSIR-Savanna Agriculture Research Institute (SARI) was sorted by hand, winnowed and milled in a disc attrition mill. Five hundred grams (500 g) of the flour was packaged in polyethylene bags. Ten of such packages of the millet flour were made, five of which were sterilized by irradiation at 5 kGy radiation dose (Mustapha et al., 2014) at the Ghana Atomic Energy Commission. The flours were used to prepare the following slurries at the pilot plant of CSIR-Food Research Institute in duplicate.

i) Spontaneous fermentation of non-sterile millet slurry.
ii) Fermentation by inoculum enrichment of non-sterile millet slurry.
iii) Spontaneous fermentation of sterile millet slurry
iv) Fermentation by starter culture of sterile millet slurry

For the preparation of a slurry, 500 g of millet flour was dissolved in 4.5 L of water to give a 10 % (w/w) slurry. Unfermented millet slurry and unfermented sterile millet slurry were also prepared. Apart from the unfermented millet slurry, spontaneously fermented millet slurry and inoculum enrichment fermentation in which tap water was used to prepare the slurries, boiled water which has been cooled was used in the preparation of all sterile millet slurries.

For the preparation of starter culture, 100 µl of LAB broth culture (glycerol stocks) was inoculated singly into 10 ml of MRS broth (Oxoid CM361) and incubated at 30 °C for 16 -18 h. Hundred microliters of the culture was transferred into another 10 ml of MRS broth and incubated for 16-18 h. Hundred microliter of the culture was transferred into another 100 ml of MRS broth and incubated for 16-18 h under agitation. The culture was transferred into 50 ml falcon tubes,

centrifuged at 4000 rpm for 10 min and the supernatant discarded. The cells were then washed with 20 ml of sterile Salt Peptone Solution (SPS) of pH 7.2. The washing procedure was carried out three times, the cells (pellets) pulled together and resuspended in 10 ml sterile distilled water. This preparation was used for the inoculation of millet slurry in a ratio of 1:1:1 immediately to achieve cell concentration of 10^6 for LAB or 10^5 for yeast. The same procedure was used for the preparation of yeast cells except that the cells were grown in Malt Extract broth (Oxoid CM57) and incubated overnight at 25 ºC.

Samples of the four different fermented slurries produced (end products) and the two unfermented slurries were analysed for pH, titratable acidity, moisture, ash, fat, protein, carbohydrate, energy, starch, iron, calcium and phosphorus contents. Samples of the fermented slurries were also taken for microbiological analysis to assess their safety for consumption. The microbiological analysis carried out were determination of Enterobacteriaceae, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus* and *Salmonella* spp.

7.2.2 Upscaling: Semi-industrial scale production of *Hausa koko* flour at a Small and Medium Scale Enterprise using starter culture RFCH

7.2.2.1 Preparation of starter culture

Limosilactobacillus reuteri LDOD-Sud, and *Limosilactobacillus fermentum* LMAN-Sdb, and the yeast *Saccharomyces cerevisiae* YSUN-Sud cultures were grown overnight singly by inoculating 100 µl of glycerol stocks in 5 ml MRS (Oxoid CM361) and Malt Extract (Oxoid CM57) broths and incubated at 30 and 25 ºC respectively for 16-18 h under agitation. The culture (5 ml) was transferred into a 100 ml of each respective growth media and incubated appropriately for 16-18 h under agitation. 25 ml of each culture was transferred into four different 1.5 L respective broth.

Thus each 100 ml culture was transferred into 6 L of respective broth. The cultures were incubated again for 16-18 h under agitation to obtain a cell concentration of 10^9 CFU/ml for LAB and 10^8 CFU/ml for yeast.

Each culture was transferred into several 50 ml falcon tubes aseptically, centrifuged at 4000 rpm for 10 min, supernatant discarded, the different sediments/pellets of a batch pulled together and washed three times with 20 ml sterile Salt Peptone Solution (SPS) of pH 7.2 to get rid of the media. The cells were resuspended in 30 ml sterile distilled water for immediate inoculation or preserved in 30 ml SPS at 4 ºC for later use (not more than two weeks).

RFCH (*L. reuteri* LDOD-Sud, *L. fermentum* LMAN-Sdb, *S. cerevisiae* YSUN-Sud) starter culture was introduced following the procedure described in Figure 22. The LAB and yeast were inoculated into 10 L of water to achieve a final population of 10^6 and 10^5 CFU/ml respectively for kneading 60 kg of millet flour into dough and fermented at room temperature (28-30 °C).

7.2.2.2 Semi-industrial scale production of *Hausa koko* at the SME using starter culture RFCH

The procedure that was used to produce *Hausa koko* flour at the SME is shown in Figure 22.

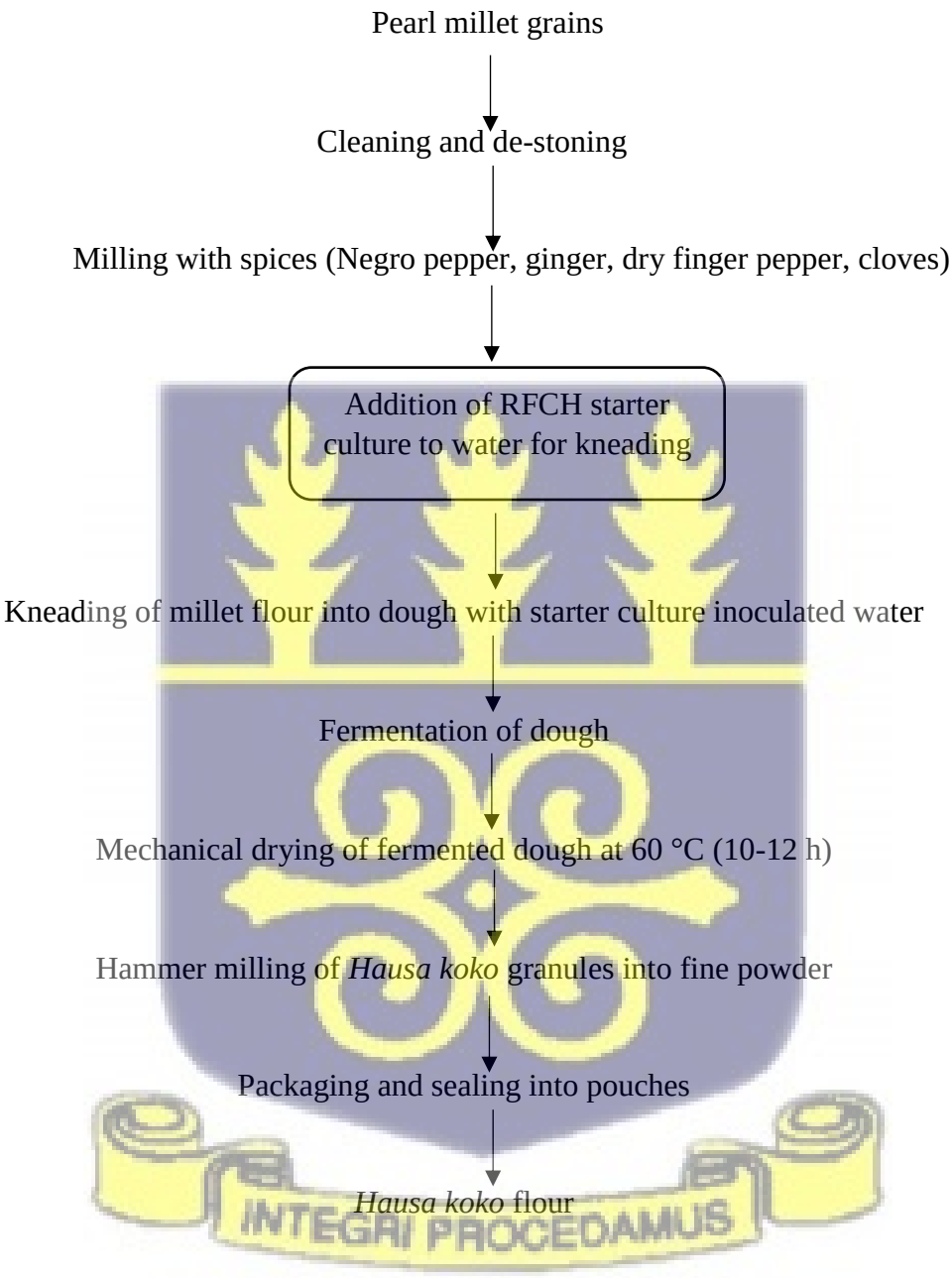


Figure 22: Flow diagram for semi-industrial scale production of *Hausa koko* flour using starter culture RFCH at the SME

This procedure used at the SME is a modification of the traditional home process. In the traditional process, after cleaning and destoning millet, the grains are steeped in water overnight, drained, and milled together with spices into a meal before it is kneaded with a little water into a dough and left to ferment for 48-72 hours. The fermented dough is then mixed with the right proportion of water and cooked into the porridge, *Hausa koko.* At the SME, steeping of the millet grains (60 kg) after manual cleaning and de-stoning was avoided altogether. The cleaned millet is milled together with washed (5 % salt solution) spices (ginger (1 kg); dry finger pepper (500 g), Negro pepper (200 g), cloves (100 g)) and kneaded into a dough which is then allowed to ferment for 48-72 hours. The dried granules are milled in a plate/hammer mill and packaged for sale in the shops. The consumer prepares the *Hausa koko* by mixing the flour with water and cooking into the breakfast porridge or following the cooking instructions on the package. This procedure was used at the SME except that the starter culture was added to water (10 L) for kneading the milled millet into dough to give an initial concentration of 10^7 CFU/g in the dough. The dough was then allowed to ferment at ambient temperature (ca 30 ^{o}C). After only 12 hours of fermentation, results (pH and titratble acidity) showed that the fermentation of the dough using Starter Culture RFCH as inoculum enrichment was okay and could be halted. However, part of the dough was allowed to ferment further. During the trial production, another batch of *Hausa koko* flour was produced at the same time but without the starter culture RFCH as control. This batch needed to be fermented for 48 h before the fermentation was satisfactory. The workers at the SME followed basic Good Manufacturing and Good Hygienic Practices during the experiment. These included washing of hands and utensils thoroughly with soap and clean water before use, hygienic handling of raw material, wearing of clean uniforms, hairnets, avoidance of talking, chewing, and touching the skin. The milling machine was also adequately cleaned with soap, sponge and hot water to remove accumulated grain flour and possible harboring microorganisms from the previous millet milled.

In the SME trial production, duplicate samples (about 500 g each) were taken at 4 h intervals for both the starter culture and spontaneous fermentations for determination of pH and titratable acidity. Samples of the doughs taken were also assessed for their microbiological quality involving determination of counts of lactic acid bacteria, yeasts, Enterobacteriaceae, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus* and *Salmonella* spp.

7.2.3 Analytical methods

7.2.3.1 Chemical methods

The physico-chemical quality was estimated by determining the pH, Titratable acidity, moisture, crude fat, crude protein, ash, energy, carbohydrate and starch using standard methods described by Pearson's Composition and Analysis of Foods, 9th Ed. Association of Official Analytical Chemists (AOAC) international methods. Iron and phosphorus were determined using UV-visible spectrophotometer at 520 nm and at 650 nm wavelength respectively whilst calcium was determined using titration method (AOAC 4.8.03) as described below.

7.2.3.2 pH

The pH of liquid samples (20 ml) were taken directly after homogenization whilst solid samples (10 g) were homogenised with 20 ml of sterile ultrapure water and determined using pH meter (CP-511 Elmetron, Poland) after calibration with standard buffers.

7.2.3.3 Titratable acidity

Ten millilitres (10 ml) of sample was topped up to 200 ml of distilled water and homogenized. Out of this, 80 ml was measured and titrated against 0.1 m NaOH using 1 % freshly prepared phenolphthalein indicator. One millilitre of 0.1 NaOH titre used was equated to 0.009 g of lactic

acid according to Amoa-Awua et al., (2007). For dough samples, 10 % slurry was prepared and used.

7.2.3.4 Moisture

The moisture content was estimated in duplicates using the air-oven at 105 ºC for 4 h and loss in weight was used in calculating the moisture content of the samples according to the method described by the modified AOAC (2016 20th Ed), method No 32.1.03

7.2.3.5 Ash

The ash content was estimated according to AOAC (2016 20th Ed), method No. 32.1.05. The sample (5 g) was weighed into a previously conditioned (ignited, cooled and weighed) porcelain ashing crucible. It was then incinerated in a vecstar furnace for 8 h at 550 ºC ± 10 ºC until the sample turned into a grey ash. It was cooled in a desiccator at room temperature, weighed and ash value calculated accordingly to the method.

7.2.3.6 Crude Fat

Crude fat was estimated according to Werner Scmid (Acid Digestion Method). 5 g (to the nearest mg) of sample was weighed into a 50 ml beaker and digested using (1+1) N HCl on a hot plae for 15 min. The digested sample was transferred into a separating funnel and 10 ml of ethanol added with 25 ml of petroleum ether plus diethyl ether and extracted 3 times into a pre-weighed conical flask. This was evaporated and dried in an oven at 105 ± 3 ºC for 1 h. The flask was then weighed and the difference in weight used in calculating the percentage fat accordingly.

7.2.3.7 Protein

Protein was determined by weighing 0.25 g of homogenised sample into a Kjeldahl flask, 3.5 g of Kjetabs catalyst tablet (mixture of copper sulphate and potassium sulphate) and 15 ml of concentrated H_2SO_4 added and swirled to mix thoroughly. They were then digested in a digester at 400 ± 10 ºC for 90 min. The distillation unit of the Kjeltec was used to distil nitrogen from the sample into a conical flask containing 25 ml of boric acid (4 %) solution containing mixture of methyl red and bromocresol green as an indicator. It was then titrated against standardised 0.1 N HCL. Using a conversion factor of 6.25, the percentage of protein was calculated according to the method described by AOAC (2016 20th Ed), method No. 4.2.09.

7.2.3.8 Carbohydrate

The carbohydrate (including fibre) content was estimated using By-difference (Atwater) method (Pearson's 1995, 9th Ed). The calculation was as follows:

% carbohydrate (including fibre) = 100 - (% Moisture + % Ash + % Fat + % Protein)

7.2.3.9 Energy

The energy content was estimated using Atwater factor calculation from the results of the proximate analyses of the samples in accordance with AOAC (2016 20th Ed). The calculation was as follows:

Energy (kcal/100g) = (% Carbohydrate x 4) + (% Protein x 4) + (% Fat x 9)

7.2.3.10 Iron

Using 2, 2-bipyridyl colorimetric method, iron was determined by pipetting 5 ml of the sample filtrate into 50 ml volumetric flask and a pinch of ascorbic acid added. The mixture was allowed to stand for 10 min, 10 ml of 20 % ammonium acetate was added followed by 2 ml of 0.2 % of 2, 2-bipyridine and observed for a light pink colour formation. This was kept in the dark for 1 h, topped up with distilled water to the 50 ml mark of the volumetric flask. It was then analysed using UV-visible spectrophotometer at 520 nm and iron content calculated accordingly.

7.2.3.11 Calcium

Calcium was estimated by pipetting 20 ml of the sample filtrate into a beaker, 10 ml of (1+1) N HCl added, 4 drops of 1 % methyl red and heated for 15 min to boil on a hot plate. This was then followed by the addition of (1+1) N NH_4OH dropwise to obtain a pH of 5.6 shown by a brownish – orange colour. 15 ml saturated Ammonium Oxalate followed by 5 g urea were also added. The solution was allowed to stand for 4 h to precipitate, filtered using Whatman paper No. 1, tested for white precipitation of Cl^- ions with HNO_3 followed by $AgNO_3$ and washed to the 300 ml mark on the conical flask with distilled water. The filter paper was transferred into a beaker and crushed in 50 ml 2N H_2SO_4, placed on hot plate to boil and titrated against 0.02N $KMn2O_4$ to obtain a faint pink colouration and calcium content calculated according to AOAC 2005, method 4.8.03.

7.2.3.12 Phosphorus

Phosphorus content was determined by pipetting 1 ml of the filtrate into 50 ml volumetric flask, 5 crystals of ascorbic acid followed by 5 ml of ammonium molybdate sulphuric acid were added. The flask was then placed in 100 ºC water bath until the content turned blue. It was then topped

up with distilled water to the 50 ml mark, analysed using UV-visible spectrophotometer at 650 nm and phosphorus content calculated according to slightly modified AOAC 2005, method 3.4.11.

7.2.4 Enumeration of microorganisms

Samples (10 g) were diluted with 90 g of Salt Peptone Solution (SPS) containing 0.1% peptone and 0.85% NaCl of pH 7.2 (Lad Blender) and homogenised for 30 sec at normal speed. The homogenate was serially diluted, 1 ml aliquot pipetted into Petri dishes and suitable isolation media poured or used to streak for enumeration using standard methods. These were: Enterobacteriaceae (NMKL 144, 2005); moulds (International Standards Organization Method (ISO) 21527-1:2008); *S. aureus* (NMKL Method No. 66, 2003); *E. coli* (NMKL. No. 125, 2005); *B. cereus* (NMKL No. 67, 2010); and *Salmonella* spp (NMKL method No. 71, 1999) as described earlier (Chapter 4; 4.2.3) according to NMKL methods. Lactic acid bacteria was enumerated by pour plate method using MRS agar (Oxoid CM361, Oxoid Ltd., UK) pH 6.2, containing 0.1 % cycloheximide supplement (Atter et al., 2014). The plates were incubated anaerobically in an anaerobic jar at 30 °C for 72 h. Yeast was also enumerated by pour plate method using Malt Extract Agar (Oxoid CM59, Oxoid Ltd., UK) containing chloramphenicol (C0113.0025, Netherlands). The plates were incubated at 25 °C for 72 h (Atter et al., 2014).

7.2.5 Sensory Evaluation of Semi-industrial Scale Inoculum Enrichment Produced *Hausa koko* Flour

The packaged RFCH inoculum enrichment fermented *Hausa koko* flour and spontaneously fermented *Hausa koko* flour were cooked into porridges following same method. Each was prepared by mixing 200 g of the flour with 1L of water in a cooking utensil, placed on fire and cooked for 25 min in total whilst stirring continuously into a smooth flowing porridge. Sugar (100

g) was added to taste for each preparation. The two porridges were assessed for acceptability by a sensory panel of 20 untrained people who were already very familiar and regular consumers of *Hausa koko*. These were staff of CSIR-Food Research Institute who were involved in the sensory evaluation at the laboratory scale studies involving the different *Hausa koko* samples prepared with the different starter culture combinations (chapter 6). The randomly coded samples using three figures were assessed based on overall acceptability on a nine-point hedonic scale ranging from 1 meaning dislike extremely to 9 meaning like extremely as shown below:

9 Like Extremely
8 Like Very Much
7 Like Moderately
6 Like Slightly
5 Neither Like nor Dislike
4 Dislike Slightly
3 Dislike Moderately
2 Dislike Very Much
1 Dislike Extremely

7.2.6 Data analysis

Data obtained were subjected to Analysis of Variance (ANOVA) and Duncan test (SPSS version 21.0). A significant level of $P \leq 0.05$ was used.

7.3 Results

7.3.1 Changes in the composition of millet slurry during fermentation with starter culture on a pilot scale.

Table 22 shows the changes in pH and titratable acidity when millet slurry was allowed to ferment spontaneously for 12 h or was allowed to ferment after the addition of Starter RFCH as inoculum enrichment. The table also shows the changes in pH and tiratable acidity when the millet slurry was first sterilized and allowed to ferment either spontaneously or by fermentation with Starter RFCH. The results show that spontaneous fermentation of the non-sterile millet slurry reduced the pH to 5.71 in 12 hours, whilst addition of the starter culture as inoculum enrichment reduced the pH to a much lower value, 3.50 within the same period. Spontaneous fermentation of sterile millet slurry for 12 h did not show any significant change in pH within the 12 h, but addition of the starter culture reduced the pH to 3.54 after 12 hours. Thus, through the use of Starter RFCH as either starter culture or inoculum enrichment achieved the range of pH and percentage titratable acidity normally recorded after 48 to 72 hours of millet fermentation in the traditional process which is also replicated by the SMEs. The results of the percentage titratable acidity corresponded to changes in pH as higher percentage titratable acidity was recorded for the lower pH values.

Table 22. Changes in pH and titratable acidity of millet slurry during fermentation with Starter RFCH on pilot scale

Sample	pH	% Titratable Acidity
Millet slurry (unfermented)	6.21 ± 0.01^{f}	0.09 ± 0.01^{e}
Spontaneously fermented millet slurry	5.71 ± 0.01^{d}	0.28 ± 0.01^{d}
Fermented millet slurry by inoculum enrichment	3.50 ± 0.01^{b}	0.48 ± 0.01^{a}
Sterile millet slurry (unfermented)	6.02 ± 0.01^{e}	0.10 ± 0.01^{e}
Spontaneously fermented sterile millet slurry	5.92 ± 0.01^{c}	0.37 ± 0.02^{c}
Sterile slurry fermented with starter culture	3.54 ± 0.01^{a}	0.41 ± 0.02^{b}

Note: Figures are presented as means ± standard deviations. Superscript to figures implies significant or not significant at $P \leq 0.05$ (ANOVA, Duncan test).

Table 23 shows the changes in the proximate composition of millet slurry during fermentation with starter culture on a pilot scale. The samples analysed were sterile and non-sterile millet slurry, spontaneously fermented sterile and non-sterile millet slurry, starter culture fermentation of sterile millet slurry and inoculum enrichment of non-sterile millet slurry. Though the differences in the value for the various components between the various samples appeared small, they were significantly different in a lot of the cases. The samples which shared the closest values for the various components, moisture, ash, fat, protein carbohydrate and energy were the unfermented millet slurries, that is, the sterile and non-sterile slurries. The only difference between these two samples was that, one had been irradiated whilst the other had not been irradiated. The moisture content of the fermented samples ranged (g/100g) from 91.63 ± 0.11 to 92.41 ± 0.12, ash from 0.17 ± 0.11 to 0.23 ± 0.04, fat from 0.34 ± 0.14 to 0.42 ± 0.21, protein from 2.16 ± 0.06 to 3.59± 0.04, carbohydrate from 3.51 ± 0.05 to 5.49± 0.17 and energy from 31.40 ± 0.29 to 36.11 ± 0.32.

Table 23. Changes in proximate composition of millet slurry during fermentation with Starter RFCH on a pilot scale

Sample	Moisture (g/100g)	Ash (g/100g)	Fat (g/100g)	Protein (g/100g)	Carbohydrate (g/100g)	Energy (Kcal/100g)
Millet slurry (unfermented)	91.09 ± 0.08^{a}	0.47 ± 0.03^{d}	0.48 ± 0.09^{d}	2.01 ± 0.02^{b}	5.97 ± 0.16^{d}	36.11 ± 0.32^{d}
Spontaneously fermented millet slurry	91.63 ± 0.11^{b}	0.23 ± 0.04^{b}	0.42 ± 0.21^{bc}	2.24 ± 0.09^{c}	5.49 ± 0.17^{c}	34.64 ± 0.25^{c}
Fermented slurry by inoculum enrichment	92.41 ± 0.12^{d}	0.17 ± 0.11^{a}	0.34 ± 0.14^{a}	3.59 ± 0.04^{e}	3.51 ± 0.05^{a}	31.40 ± 0.29^{a}
Sterile millet slurry (unfermented)	91.51 ± 0.06^{b}	0.41 ± 0.21^{c}	0.44 ± 0.11^{cd}	1.81 ± 0.03^{a}	5.83 ± 0.05^{d}	34.52 ± 0.24^{c}
Spontaneously fermented sterile millet slurry	91.86 ± 0.11^{c}	0.21 ± 0.09^{ab}	0.41 ± 0.21^{bc}	2.16 ± 0.06^{c}	5.37 ± 0.05^{c}	33.75 ± 0.29^{b}
Sterile slurry fermented with starter culture	92.33 ± 0.08^{d}	0.19 ± 0.08^{ab}	0.39 ± 0.31^{b}	2.80 ± 0.34^{d}	4.30 ± 0.13^{b}	31.89 ± 0.30^{a}

NB: Figures are presented as means ± standard deviations. Superscript to figures implies significant or not significant at $P \leq 0.05$ (ANOVA, Duncan test)

Changes in the concentration of three minerals iron, calcium and phosphorus, in the millet slurries and various fermented slurries are given in Table 24. In all cases, there was an increase in the content of the three minerals from the millet slurry to the spontaneously fermented slurry to the slurry fermented with Starter RFCH as starter culture.

Table 24. Changes in iron, calcium and phosphorus content (mg/100g) of millet slurry during fermentation with Starter RFCH on a pilot scale

Sample: Millet slurry	**Iron (mg/100g)**	**Calcium (mg/100g)**	**Phosphorus(mg/100g)**
Millet slurry	3.07 ± 0.17^{b}	38.65 ± 1.77^{b}	8.21 ± 0.74^{b}
Spontaneously fermented millet slurry	4.80 ± 0.11^{c}	81.77 ± 0.47^{d}	10.84 ± 0.91^{c}
Fermented slurry by inoculum enrichment	5.80 ± 0.24^{d}	103.74 ± 5.85^{e}	15.01 ± 0.48^{d}
Sterile millet slurry	2.07 ± 0.13^{a}	30.35 ± 0.87^{a}	5.44 ± 0.24^{a}
Spontaneously fermented sterile millet slurry	2.99 ± 0.05^{a}	63.07 ± 0.28^{c}	8.41 ± 1.54^{b}
Sterile slurry fermented with starter culture	3.49 ± 0.08^{e}	91.99 ± 1.41^{f}	11.80 ± 0.92^{c}

Figures are presented as means ± standard deviations. Superscript to figures implies significant or not significant at $P \leq 0.05$ (ANOVA, Duncan test).

The same trend is also observed from the sterile slurry to fermentation of the sterile slurry to sterile slurry fermented with the starter culture. In each of these two cases, one would expect an increase in microbial population from the millet slurry to the spontaneously fermented slurry to the inoculum enriched/starter culture fermented slurry.

7.3.2 Microbiological safety of millet slurry during pilot scale fermentation

The presence and population of Enterobacteriaceae, *E. coli, Staphylococcus aureus*, *Bacillus cereus*, and *Salmonella* spp in the millet slurries before and after the pilot scale fermentations were determined and the results given in Table 25. None of these organisms was detected in the millet slurry fermented by inoculum enrichment, the sterile millet slurry, spontaneously fermented sterile millet slurry, and sterile millet slurry fermented with the starter culture. With the other two samples, unfermented millet slurry and the spontaneously fermented millet slurry, *E. coli* and *Salmonella* spp were not detected, however Enterobacteriaceae, *Staphylococcus aureus*, and *Bacillus cereus* were present in the samples. The population of these organisms had reduced by about 0.5 to one log unit during the spontaneous fermentation of the non-sterile millet slurry as seen in Table 26.

Table 25. Microbial quality characteristics of 12 h starter culture (RFCH) and inoculum enriched fermentation (log CFU/g) during pilot study

Sample	Enterobacteriaceae	*E. coli*	*Staph aureus*	*Bacillus cereus*	*Salmonella spp.*
Millet slurry (Unfermented)	2.59 ± 0.15	nd	1.61 ± 0.05	1.40 ± 0.11	nd
Spontaneously fermented millet slurry	1.25 ± 0.10	nd	1.18 ± 0.14	1.09 ± 0.12	nd
Fermented slurry by inoculum enrichment	nd	nd	nd	nd	nd
Sterile millet slurry (Unfermented)	nd	nd	nd	nd	nd
Spontaneously fermented sterile millet slurry	nd	nd	nd	nd	nd
Sterile slurry fermented with starter culture	nd	nd	nd	nd	nd

NB: Each value represents the mean log and standard deviation of samples; nd = not detected.

7.3.3 Acidification of millet dough during semi-industrial scale production of *Hausa koko* flour at a Small and Medium Scale Enterprise in Accra

Only two procedures for fermentation of millet were tried on semi-industrial scale since large scale sterilization of millet grains was not feasible at the SME. Thus, only non-sterile millet grains were used as in the traditional process; and also, the starter culture could only be used as inoculum enrichment since the millet was non-sterile and contained its natural microbiota. Changes in pH and titratable acidity during the control spontaneous fermentation and the inoculum enrichment with starter RFCH fermentation are shown in Figures 23 and 24. In the control or the normal SME fermentation, the pH at the start of fermentation was 6.01 and at the end of 48 hours had reduced to 4.42. In the novel inoculum enriched fermentation, the pH at the start of fermentation was 5.64 and by the 12th hour had already significantly ($P \leq 0.05$) reduced to 4.43, which was even slightly higher than the final pH attained in the spontaneous 2 days fermentation. Part of the inoculum

enriched fermentation was allowed to ferment for 2 days also and recorded an even lower pH of 3.41 compared to the pH of 4.42 in the 2 days spontaneous fermentation. With regards to percentage titratable acidity, the value at the start of the spontaneous fermentation was 0.15 % compared to 0.3 % in the inoculum enriched fermentation. The titratable acidity at the end of the 2 days spontaneous fermentation was 1.14 %, a concentration which was attained in the inoculum enriched fermentation after only 12 hours. When part of the inoculum enriched dough was allowed to ferment till the end of 2 days, the percentage titratable acidity was 1.61 %. At 12 h of the inoculum enriched fermentation it was assessed that the fermentation was okay based on results obtained. Part of the batch was therefore processed further into the *Hausa koko* flour, though some of the dough was allowed to ferment further in line with the control sample. Thus, through the use of Starter RFCH as inoculum enrichment, the fermentation of millet during semi-industrial scale *Hausa koko* production could be reduced from 48 to 12 hours.

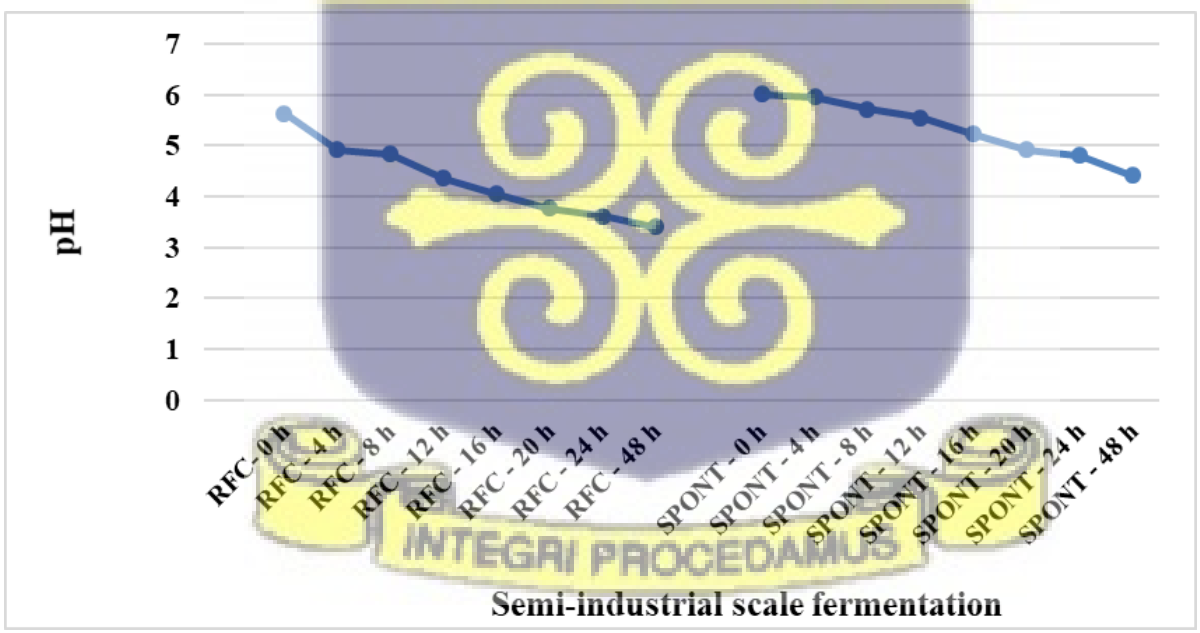


Figure 23: pH values of inoculum enriched starter culture (RFCH) and spontaneous fermentation at a semi-industrial scale production site

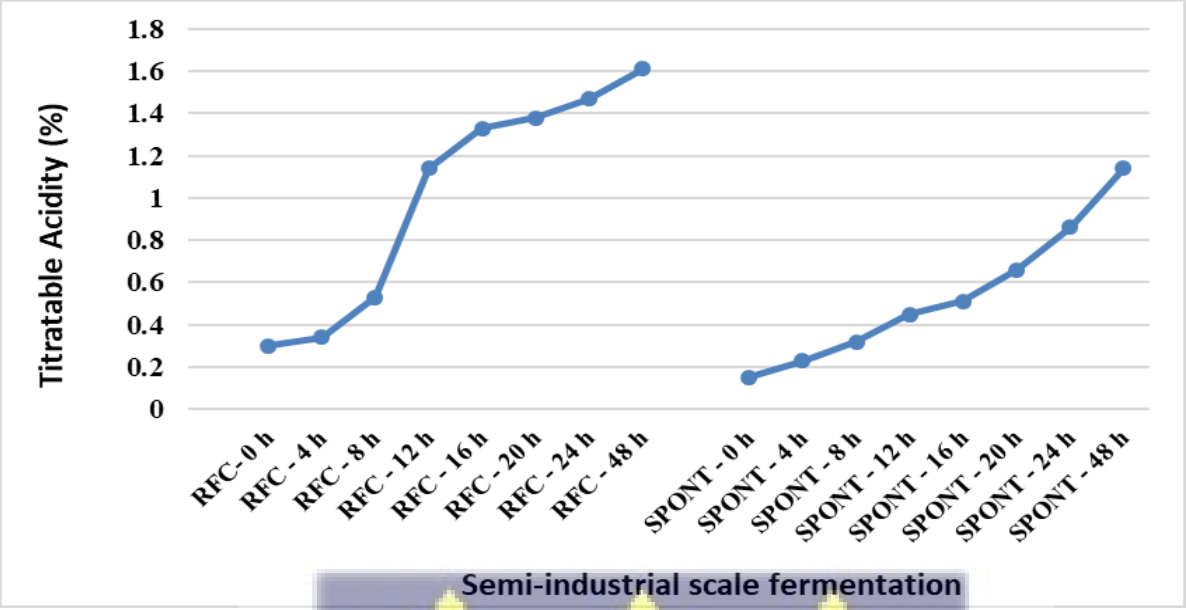


Figure 24: Titratable acidity of inoculum enriched starter culture (RFCH) and spontaneous fermentation at a semi-industrial scale production site for the 48-h duration.

NB:

RFC = *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud; (RFCH Starter Culture)
SPONT = Spontaneous fermentation

7.3.4 Changes in microbial population during semi-industrial scale production of *Hausa koko* flour at a Small and Medium Scale Enterprise in Accra

The microbial population of the fermenting millet dough samples were analysed during the semi-industrial scale production of *Hausa koko* flour at the SME and is shown in Table 26. Generally, the population of lactic acid bacteria and yeasts in the novel inoculum enriched fermentation were 2 log units higher than in the spontaneously fermented dough. The higher population of LAB and yeasts during the inoculum enrichment fermentation could be attributed directly to the additional cells of *Limosilactobacillus reuteri, Limosilactobacillus fermentum* and the yeast *Saccharomyces*

cerevisiae which were added as the starter culture. The population of lactic acid bacteria at the start of dough fermentation in the spontaneous or the normal SME sample was log 4.38 CFU/g while it was log 7.83 CFU/g in the inoculum enriched sample (Table 26). With yeasts, the population in the spontaneous fermentation was log 3.66 CFU/g at the start of fermentation and log 6.63 CFU/g in the inoculum enriched fermentation. The differences in concentration of the lactic acid bacteria and yeasts could be attributed directly to the addition of RFCH Starter Culture as inoculum enrichment. At 12 hours of fermentation, the LAB and yeasts populations in the inoculum enriched fermentation had increased to log 10.77 CFU/g and 7.85 CFU/g, respectively. The metabolic activities of this high population of lactic acid bacteria had already resulted in the production of 1.14 % lactic acid, reducing the pH to 4.43 and the fermentation could be terminated after only 12 hours compared to the usual 48 to 72 hours of spontaneous fermentation. The LAB and yeasts populations in the spontaneously fermented dough at the end of 48 hours when it was okay for further processing were log 8.75 CFU/g and 6.79 CFU/g respectively.

Table 26. Changes in microbial population (log CFU/g) during fermentation of millet dough in the semi-industrial scale production of *Hausa koko* flour at a Small and Medium Scale Enterprise in Accra

Sample	LAB	Yeast	Moulds	Enterobact.	*Staph. aureus*	*Bacillus cereus*	*E. coli*	*Salmonella* spp
Spontaneous fermentation								
0 h	4.38 ± 0.09^{a}	3.66 ± 0.03^{a}	2.66 ± 0.04^{c}	5.89 ± 0.05^{i}	4.95 ± 0.09^{k}	3.87 ± 0.02^{k}	3.95 ± 0.05^{j}	nd^{a}
4 h	4.67 ± 0.05^{b}	3.93 ± 0.02^{b}	2.61 ± 0.04^{c}	5.86 ± 0.02^{li}	4.92 ± 0.03^{k}	3.86 ± 0.03^{jk}	3.95 ± 0.02^{j}	nd^{a}
8 h	$5.62 \pm 0.05c$	4.48 ± 0.05^{c}	2.58 ± 0.05^{c}	5.83 ± 0.03^{i}	4.85 ± 0.02^{j}	3.74 ± 0.03^{i}	3.85 ± 0.02^{i}	nd^{a}
12 h	6.84 ± 0.03^{d}	4.73 ± 0.03^{d}	1.48 ± 0.05^{b}	4.96 ± 0.02^{h}	3.74 ± 0.03^{g}	3.54 ± 0.07^{h}	3.58 ± 0.06^{h}	nd^{a}
16 h	6.97 ± 0.02^{e}	4.97 ± 0.08^{e}	1.29 ± 0.09^{b}	4.92 ± 0.02^{h}	3.71 ± 0.04^{g}	2.93 ± 0.01^{g}	2.88 ± 0.02^{g}	nd^{a}
20 h	7.80 ± 0.03^{f}	5.60 ± 0.05^{f}	1.42 ± 0.04^{b}	4.78 ± 0.06^{g}	3.56 ± 0.06^{f}	2.65 ± 0.03^{f}	2.73 ± 0.03^{f}	nd^{a}
24 h	7.93 ± 0.03^{f}	5.86 ± 0.02^{g}	nd^{a}	4.35 ± 0.10^{e}	2.94 ± 0.01^{e}	2.30 ± 0.07^{e}	2.57 ± 0.04^{e}	nd^{a}
Inoculum enrichment with Starter RFCH								
0 h	7.82 ± 0.04^{f}	6.62 ± 0.05^{h}	2.58 ± 0.04^{c}	5.84 ± 0.02^{i}	4.65 ± 0.04^{i}	3.77 ± 0.04^{ik}	3.67 ± 0.05^{h}	nd^{a}
4 h	7.95 ± 0.08^{f}	6.75 ± 0.04^{i}	1.44 ± 0.08^{b}	4.58 ± 0.07^{f}	3.84 ± 0.03^{h}	2.64 ± 0.04^{f}	2.93 ± 0.03^{g}	nd^{a}
8 h	9.88 ± 0.03^{h}	7.77 ± 0.03^{j}	1.38 ± 0.05^{b}	2.91 ± 0.03^{c}	2.49 ± 0.04^{d}	1.41 ± 0.07^{c}	1.83 ± 0.03^{d}	nd^{a}
12 h	10.76 ± 0.04^{i}	7.85 ± 0.03^{j}	nd^{a}	1.43 ± 0.07^{b}	1.26 ± 0.04^{b}	1.15 ± 0.10^{b}	1.45 ± 0.05^{c}	nd^{a}
16 h	10.87 ± 0.07^{ij}	7.97 ± 0.07^{k}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
20 h	10.97 ± 0.02^{j}	8.39 ± 0.09^{l}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
24 h	11.17 ± 0.14^{k}	8.89 ± 0.02^{m}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
48 h	11.30 ± 0.13^{k}	9.92 ± 0.06^{n}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}

NB: Each value represents the mean log and standard deviation of samples and superscript to values implies significant or not significant at $P \leq 0.05$ (ANOVA, Duncan test).

Enterobact. = Enterobacteriaceae

Staph. aureus = *Staphyloccus aureus*

nd = not detected

One of the factors used in developing the starter culture, was the antimicrobial activities of the lactic acid bacteria including their potential for producing bacteriocins based on the presence of bacteriocin producing genes in their genome. The presence of some indicator organisms and common foodborne pathogens were therefore monitored during the semi-industrial scale fermentations to assess the impact of Starter RFCH on the safety of *Hausa koko*. The organisms monitored were Enterobacteriaceae, *E. coli*, *Staphylococcus aureus*, *Bacillus cereus*, and *Salmonella* spp and their counts during the two fermentations are shown in Table 26.

At the start of the two semi-industrial scale fermentations, that is, spontaneous and inoculum enriched fermentations, both millet doughs had very similar concentrations of these organisms, apart from *Salmonella* spp which was not isolated during any of the fermentations. Also, the levels of these organisms in the doughs were high. The levels of these organisms at the start of both fermentations were Enterobacteriaceae - 10^6 CFU/g, *E. coli* - 10^4 CFU/g, *Staphylococcus aureus* - 10^5 CFU/g, and *Bacillus cereus* - 10^4 CFU/g. In the spontaneous or normal fermentation at the SME, the population of the above listed organisms had reduced by one or less than one log unit after 12 hours of fermentation. However, in the novel inoculum enriched fermentation the reductions recorded were between 3 and 5 log units. After 16 h of fermentation, none of these organisms were detected in the inoculum enriched fermentation. In the spontaneous fermentation even after the 48 hours of fermentation, the population of these organisms were Enterobacteriaceae, log 3.83. CFU/g; *E. coli* log 1.30 CFU/g; *Staphylococcus aureus* log 4.95 CFU/g; and *Bacillus cereus* log 1.60 CFU/g.

7.3.5 Sensory evaluation of semi-industrial scale RFCH starter culture produced *Hausa koko* flour

The mean scores of ratings for laboratory based acceptability test by the 20 member panel of the semi-industrial scale RFCH inoculum enrichment fermented *Hausa koko* and the spontaneously fermented one on a 9 point hedonic scale are shown in Table 27. The results showed that the porridge fermented by inoculum enrichment with *L. reuteri* LDOD-Sud + *L. fermentum* LMAN-Sdb + *S. cerevisiae* YSUN-Sud was scored highest in all attributes. This sample scored highest mean value in appearance, aroma, consistency, taste and overall acceptability as compared to the spontaneously fermented sample. The scores for appearance ranged between 7.50 and 7.72 (liked moderately); aroma between 7.46 and 8.09 (liked moderately to liked very much); consistency between 7.00 and 7.18 (liked moderately); taste between 7.09 and 8.46 (liked moderately to liked very much) and overall acceptability between 7.55 and 8.32 (liked moderately to liked very much). There were significant differences ($P \leq 0.05$) in the mean scores for aroma, taste and overall acceptability with no significant differences ($P \leq 0.05$) in the mean scores for the other sensory attributes amongst the two products. The most preferred was the inoculum enriched fermented *Hausa koko*. A web chart (Figure 25 using XLSTAT version 2019.0.1 of the results showed distinct differences in the two samples with the inoculum enriched porridge showing higher mean scores for taste, aroma and overall acceptability.

Table 27: Sensory results of semi-industrial scale RFCH inoculum enrichment produced *Hausa koko*

Sample	Appearance	Aroma	Consistency	Taste	Overall Acceptability
Starter RFCH	7.72 ± 0.94[a]	8.09 ± 0.68[a]	7.18 ± 1.10[a]	8.46 ± 0.67[a]	8.32 ± 0.78[a]
Spontaneous fermentation	7.50 ± 0.96[a]	7.46 ± 0.86[b]	7.00 ± 1.02[a]	7.09 ± 1.44[b]	7.55 ± 0.74[b]

NB: Means across a column with different letters are significantly different at $P \leq 0.05$

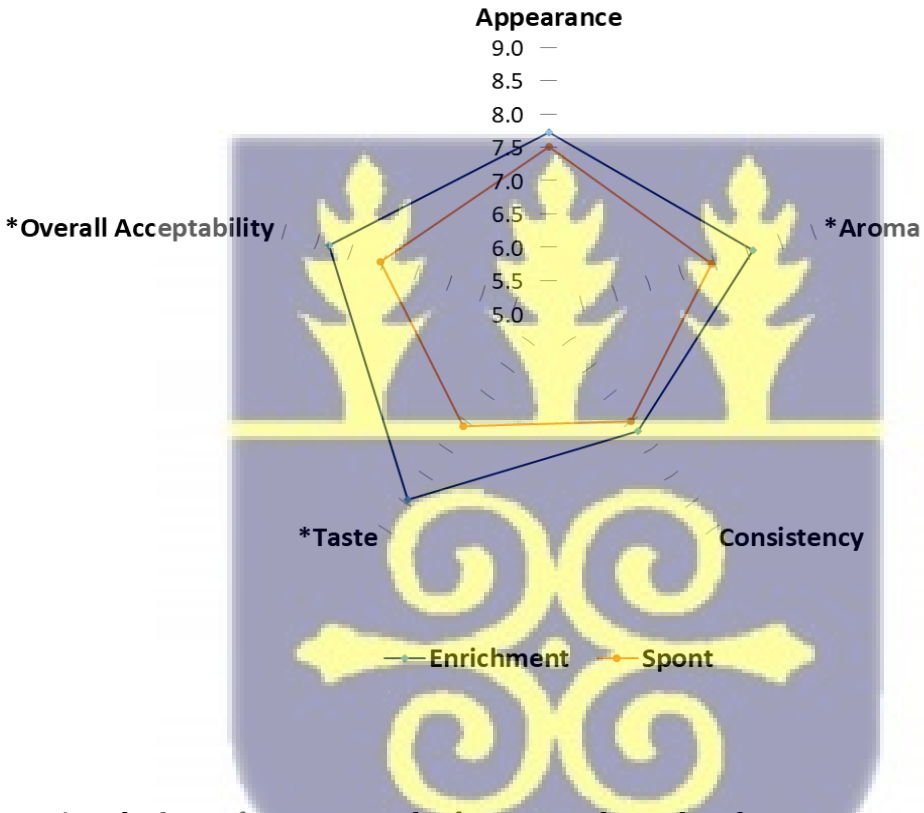


Figure 25: A web chart of sensory results for semi-industrial scale spontaneous and RFCH inoculum enrichment produced *Hausa koko*.

NB: *= Significant difference; Spont = Spontaneous fermentation; Enrichment = Inoculum Enrichment

7.4 Discussion

Pilot scale fermentations of millet slurries involving Starter RFCH as either pure starter culture or inoculum enrichment recorded lower pH values with corresponding higher titratable acidity which were significantly different ($P \leq 0.05$) in comparison, to the traditional spontaneous fermentation of *Hausa koko*. The decreases in pH could be attributed to a faster production of organic acids due to a higher population of lactic acid bacteria in the Starter RFCH fermentations. Again, it is noted that the use of Starter RFCH as inoculum enrichment recorded a significantly lower pH in the 12 hours in comparison to its use as pure starter culture during the pilot study. This could be explained by higher concentration of lactic acid bacteria and yeasts in the inoculum enrichment since the natural microbiota of the millet was present in inoculum enrichment leading to a higher concentration of fermentative organisms in the inoculum enrichment fermentation.

The increase in TTA could be attributed to ascendency of LAB population in the fermenting environment resulting in increase in carbohydrate degradation for acidification (Onuoha et al., 2017; Wakil & Kazeem, 2012). These results also show that the organisms making up the starter culture, *Limosilactobacillus reuteri*, *Limosilactobacillus fermentum* and *Saccharomyces cerevisiae* are able to dominate the indigenous microbiota of millet slurry or dough during fermentation. According to Soro-Yao et al., (2014) this is a good and expected characteristic of a good starter culture.

Starter RFCH produced higher quantities of organic acids resulting in lower pH values with corresponding higher TTA values compared to the spontaneous fermentation. The reduction in the counts of these undesirable organisms in the millet slurry could be attributed to the reduction in pH during the spontaneous fermentation. A consequence of this was a faster elimination of contaminating microorganisms including Enterobacteriaceae, *E. coli*, *S. aureus* and *B. cereus*

whose presence were monitored during the pilot fermentations. These are pathogens or indicator organisms whose presence indicated poor hygienic practices as well as an indication of unsafe food and need to be eliminated from *Hausa koko* to improve its safety. As seen in the result, there was a faster rate of microbial load reduction in the RFCH starter culture fermented millet dough as compared to the spontaneously fermented dough. Reduction of pH below 4 within 24 h in starter culture cereal fermentation is critical (Soro-Yao et al., 2014). These outcomes met the expectations of using a starter culture to induce a faster consistent fermentation process (De Melo Pereira et al., 2020). At the start of the two semi-industrial scale fermentations, that is, spontaneous and inoculum enriched fermentations, both millet doughs had very similar concentrations of these organisms, apart from *Salmonella* spp which was not isolated during any of the fermentations. Also, the levels of these organisms in the doughs were high. The reasons for these trends are because both doughs were prepared from the same batches of raw materials, that is, millet, water and the various spices. Also spices sold on the open market often have high microbial loads due to poor hygienic handling such as sun-drying on the ground in the open without any protection. As such, washing in 5 % salt solution may not significantly reduce their microbial load. The initial levels of contamination was high in the dough during the semi-industrial scale production trial study, which influenced the protective ability of the starter culture, resulting in longer fermentation period than the pilot scale slurry trials which was also slightly different in procedure. This agrees with the assertion made by Young & O'Sullivan (2011) that a starter culture's protective abilities depend on the nature of the contaminating species, contamination levels from the onset, fermentation period as well as the conditions of storage. It is also suggested that because minimum volume of water was used during the dough kneading, it prevented the faster proliferation of LAB and yeast, resulting in longer time for achieving higher acidity. Potential pathogenic organisms

like *Staph. aureus*, *B. cereus* and *E. coli* were inhibited in the RFCH starter culture dough due to the faster acidification produced whilst the spontaneously fermented dough still recorded their presence. The inhibition of these potential pathogens increases the shelf life of the product and improves its microbial safety (Ekwem 2014; Okerere et al., 2012).

The differences in the value for the various components between the various samples appeared small but they were significantly different ($P \leq 0.05$) in a lot of the cases during the pilot scale fermentation. The samples that shared the closest values for the various components, moisture, ash, fat, protein carbohydrate and energy were the unfermented millet slurries, that is, the sterile and non-sterile slurries. The only difference between these two samples was that one had been irradiated whilst the other had not been irradiated. Significant increases ($P \leq 0.05$) were observed in moisture, protein, iron, calcium and phosphorus levels during pilot scale fermentation of the millet slurries, whilst carbohydrate, fat and energy decreased. These effects were greater in the fermentations in which Starter RFCH was used either as pure starter culture or as inoculum enrichment, in comparison to the spontaneous fermentation. The inoculum enriched fermentation showed the highest increases of these constituents and may be due to a higher microbial population since these two fermentations were inoculated with the same concentrations of Starter RFCH. In the inoculum enrichment, the same concentration of the Starter RFCH added was additional to the natural microbiota in the millet slurry, hence had a higher microbial population than the pure starter culture fermentation. Increase in moisture in both spontaneous and starter culture fermented slurries have been reported for other pearl millet fermentations (Ojokoh et al., 2015). Similarly, Onuoha et al., (2017) reported increase in moisture content and protein levels after fermentation of peal millet. David & Aderibigbe (2010) have suggested that slight increases in moisture during

such fermentations may be the result of increased microbial population which boosts the breakdown of substrates and as a result released more water.

Apena et al. (2015) have described fermentation as a process for improving the protein levels in fermented foods, and for improving the amino acid balance, and their functionality. Production of extracellular enzymes and their subsequent proteolytic activities is responsible for the increase in protein content during fermentation (Apena et al., 2015; Amankwah et al., 2009). Amankwah et al., (2009) reported increase in protein content during maize flour fermentation. Adiandri and Hidayah (2019) have reported increase in protein content after *L. casei* fermentation of sorghum flour. Fasasi (2009) has also reported that in pearl millet germination and fermentation processes increased the protein content.

With the fermented samples, changes brought about by the microorganisms during the 12 hours of fermentation as well as the content from their cells would account for the differences observed. Cereal fermentation decreases the levels of anti-nutritional compounds such as phytates, tannins and phenols, which form complexes with available minerals (Rodríguez et al., 2009; Songré-Ouattara et al., 2008). These decreases in effect cause an increase in the minerals content and could explain the increments observed in iron, calcium and phosphorus levels in the present study. Also, the increase in microbial population is likely to be the main reason for the increase in the minerals content recorded due to contribution of the minerals from the microorganisms. Adiandri & Hidayah (2019) have reported that the use of *L. casei* for sorghum flour fermentation decreased tannins level after 8 h and further after 12 h. Decreases in tannins level increased with increased concentrations of starter culture and period of fermentation.

In the present study, decreases were observed in ash, fat, carbohydrate, and energy during the pilot scale fermentation of millet slurries. The reduction in ash content in the fermented slurries could be attributed to their usage by the fermenting organisms during metabolism (Nnam, 2001). The reduction in fat content was greater in the starter culture fermentations either as pure culture or inoculum enrichment in comparison to the spontaneous fermentation. The reduction in fat content during fermentation may be due to utilization of the fat by the fermenting organisms (Babalola & Giwa, 2012). Onuoha et al., (2017) have reported similar findings and suggested that it could reduce rancidity and increase the shelf life of the product.

In the fermentation of the slurries, a reduction was recorded in carbohydrate content. This would have resulted from utilization of simple or fermentable sugars by the lactic acid bacteria and the yeasts. *Saccharomyces cerevisiae* for example would have fermented glucose, maltose and maltotriose present into ethanol and carbon dioxide under anaerobic conditions of limited oxygen whilst the lactic acid bacteria would have metabolized glucose molecules present for energy. The highest reduction in carbohydrate was recorded for the slurry fermented by inoculum enrichment with the starter culture. The LAB organisms making up the starter culture are amylolytic, hence utilized starch in the millet. Again, in the natural flora of the millet present in the spontaneous and inoculum enriched fermentations, there was likely to be amylolytic organisms present which would have also broken down the starch into the simpler fermentable sugars. These would then have been utilized by the starter culture organisms, hence the highest reduction of carbohydrate recorded in the fermentation by inoculum enrichment. During fermentation, LAB and yeast activities need a lot of nutrients and energy which resulted in decrease in carbohydrate and energy as observed in this study (Simwaka et al., 2017).

In the present work, irradiation may have contributed to some of the changes recorded in the final composition of the fermented slurries. There are diverse reports either of increases or decreases in the effect of radiation on cereal flours (Abdalla et al., 2015; Sokrab et al., 2012). According to Mohamed et al. (2010), radiation of millet flour decreases anti-nutritional factors and increases the extractability of minerals significantly. However, Bashir et al., (2017) have reported that proximate compositions of wheat flour did not change with different gamma radiation dosages. Also, Aziz et al., (2006) have reported that doses of 10 kGy does not adversely affect the nutritional quality of cereal grains.

Adoption of RFCH starter culture to the substrate as expected was clearly established in this pilot and semi-industrial scale trial study (Holzapfel, 2002). Although not under strict controlled fermentation set up, this starter culture demonstrated stability, which is another suitability criteria, for its usage for large scale production (Yao et al., 2009). It was able to dominate the indigenous microbial population of the fermenting matrix as expected (Soro-Yao et al., 2014). Together with other desirable traits, RFCH starter culture was able to improve the quality of the fermented dough and reduced fermentation time from the normal 48 to 72 h to only 12 h during semi-industrial scale fermentation at the premises of the SME *Hausa koko* flour producer. In a similar upscaling study to improve millet-based fermentation for *arraw* production in Senegal, spray dried *L. plantarum* was demonstrated as a starter culture that can be used by small-scale processors (Totté et al., 2003)

Limosilactobacillus reuteri LDOD-Sud (**R**) + *Limosilactobacillus fermentum* LMAN-Sdb (**F**) + *Saccharomyces cerevisiae YSUN-Sud* (**C**) combination used as starter culture for the fermented *Hausa koko* flour and the spontaneously fermented flour was prepared into porridge and assessed for consumer acceptability. A good acceptability for the inoculum enriched fermented *Hausa koko* porridge sample was shown by the 20-member panellist. They adjudged the *Hausa koko* produced

from starter RFCH fermented millet dough highest in terms of appearance, aroma, consistency, taste and overall acceptability as compared to the spontaneously fermented sample. There was improvement in terms of appearance and consistency, but these were not significant ($P \leq 0.05$). These outcomes confirms that the use of RFCH starter culture as inoculum enrichment for fermenting millet dough for *Hausa koko* improved the aroma, taste and overall acceptability. There are extensive reports on the influence of starter culture fermentation in terms of improvement on the general sensorial attributes of fermented cereals (Olojede et al., 2020; Nami et al., 2019).

7.5 Conclusion

The results of the present study have shown that RFCH Starter Culture made up of *Limosilactobacillus reuteri* LDOD-Sud, *Limosilactobacillus fermentum* LMAN-Sdb, and *Saccharomyces cerevisiae* YSUN-Sud is suitable for use as starter culture for fermentation of millet. Its quality and safety performance met the expectations of using a starter culture to induce a faster consistent fermentation process. The use of RFCH Starter Culture as inoculum enrichment added to water for kneading the dough by semi-industrial scale millet fermentation following same or similar production process is possible and may be recommended. The inoculation can be done during the kneading process as the steeping stage is skipped during processing. Observing simple Good Manufacturing and Good Hygienic Practices during the process to avoid contamination is key for optimum result. Due to the protocols involved in its semi-industrial scale use, it may not be suitable for use by traditional household level food processors whose operations are restricted to traditional practices. Unless the starter culture is supplied in convenient dehydrated form for easy application with some education. The use of the starter culture improves the safety of the products due to its antimicrobial activities including production of organic acids and bacteriocins.

It will assure semi-industrial scale products of consistent quality since the organisms multiply very quickly into very high numbers, thus dominating the fermentation microbiota. The use of RFCH Starter Culture also reduced fermentation time of between 48 to 72 hours to only 12 hours but can be adjusted to 24 h by reducing the quantity of the starter used for daily shifts. This will in the least double the output of SMEs engaged in the semi-industrial scale production of indigenous fermented millet foods as convenience foods. Sensory analysis showed that *Hausa koko* produced from RFCH Starter Culture fermented millet was preferred to the product from spontaneous fermentation even when used for inoculum enrichment.

CHAPTER EIGHT

8.0 General discussion, conclusions and recommendations

8.1 General discussion

Hausa koko, a spicy, spontaneously fermented pearl millet porridge is mostly eaten as breakfast in Ghana by children and adults of all social classes. It is usually prepared at the household level by women. Lei & Jakobsen (2004), described a process of *Hausa koko,* production (Figure 1 step A), which depicts the fermentation characteristics of the product. Figure 1 step B also shows the process flow of a variant preparation method this work studied. Differences include longer grain steeping and longer slurry fermentation times. The variations could likely influence the microbial diversity of the final product. In recent years, there has been a rise in semi-industrial production of some indigenous foods including *Hausa koko*. But then again, the process is still spontaneous and could result in products of varying quality and safety, and thus constitutes a limitation in the achievement of the semi-industrialization efforts. There are yet, no starter cultures to produce *Hausa koko*, and there is the need to develop them. To develop a starter culture, however, requires an in-depth appreciation of the microbial diversity of the *Hausa koko* processes across the different geographical locations. This study, therefore, was conducted to identify and characterise the LAB and yeasts associated with the processing of millet into *Hausa koko* in Ghana, using phenotypic and genotypic methods to develop starter cultures to improve its quality and safety.

To serve as a guide in the design of starter cultures, an in-depth study into the bacterial diversity of samples obtained from twelve processors from six regions of Ghana was used. These were collected at seven different stages of processing (millet grains, 12 and 24 h fermented millet, milled millet with spices, supernatant of slurry, sediment of slurry and *Hausa koko*). Bacterial diversity

was conducted using a high throughput Illumina HiSeq sequencing of the V4 hypervariable region of the 16S rRNA gene amplicons and the metabolites they produced were profiled using nuclear magnetic resonance spectroscopy (NMR). High species richness was obtained from the different stages of *Hausa koko* production which was a clear indication of the abundance and variety of bacteria that existed at the different processing stages. Over four hundred (400) different Gram positive and Gram negative bacterial were involved in the entire production process. Irrespective of the geographical location they were obtained from, the samples were generally similar at the same processing stages in terms of the bacterial diversity, which may be attributed to the processors following the same production process and the use of the same or similar raw materials. The millet grains recorded a relatively higher population of the Gram negative bacterial genus *Pantoea.* They included *Escherichia-Shigella, Enterbacteriaceae, Staphylococcus, Serratia, Pseudomonas, Chryseobacterium, bacteroides, sphingomonas* which are potential pathogens associated with soil, faecal and environmental contaminants capable of causing foodborne illnesses (Azizi et al., 2020; Gadaga et al., 2008; 2004). A major shift in the bacterial community from the grains to the fermentation stages (12 h, 24 h, M, Su and Sd) was however recorded. This was dominated by lactic acid bacteria (LAB) groups mostly *Lactobacillus (now Limosilactobacillu). Pediococcus, Weissella, Lactococcus, Streptococcus* and *Leuconostoc* were the others. *Acetobacter* and *Gluconobacter* were also present in different levels of relative abundance at all the stages of *Hausa koko* production. The shift was attributed to the lowering of the pH due to the production of organic acids by the fermenting microorganisms (Achi & Ukwuru, 2015; Owusu-Kwarteng et al., 2012). Their abundance however reduced in the *Hausa koko* samples across the six regions which might be due to higher volumes of dilution with water and heat application during cooking. Significant differences existed between different processing stages because they each carried their unique

microbes. Samples from the Northern Region were also found to be different from those from the other regions due to the presence of *L. helveticus* in them. Thirty-three (33) different metabolites in varying concentrations were also produced by the microbial community at each stage of processing. The differences recorded in types and concentrations were attributed to the composition of individual raw materials including millet, spices, water, the diversity and concentrations of microbes they carried (Akpinar-Bayizit et al., 2010; Jespersen, 2003).

LAB and yeast occur naturally in the ecological niche of cereals and play important roles during their fermentation. In-depth study of these key viable LAB and yeast isolates involved in the fermentation of millet during *Hausa koko* production was therefore necessary for further characterisation. A steady increase in the population of LAB and yeast was observed from the grains through to the fermentation stages, peaking in the supernatants and sediment samples with a decrease in pH due to the production of acidic and other antimicrobial metabolites. This however decreased in the final product, *Hausa koko*. Similar trends have been reported in many fermented foods including porridges in Africa (Houngbédji et al., 2018; Wakil & Daodu, 2011). Whole genome and Sanger sequence data were analyzed using the National Center for Biotechnology Information (NCBI) database for the genomic characterisation of LAB and yeast respectively. The most frequently occurring LAB and yeast responsible for the fermentation of millet grains and millet slurry during *Hausa koko* production were *Limosilactobacillus pontis* representing 31.11 %, *Pediococcus acidilactici* and *Limosilactobacillus fermentum*, 16.67 % each; *Pediococcus pentosaceus*, 11.11 %; *Limosilactobacillus reuteri*, 10 %; *Weissella confusa*, 6.67 %; *Schleiferilactobacillus harbinensis*, 3.33 %; *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei*, 2.22 % each. *L. pontis*, *L. fermentum*, *P. pentosaceus* and *L. reuteri* occurred at all the stages of *Hausa koko* production. *Saccharomyces cf. cerevisiae/paradoxus* (41.4 %),

Saccharomyces cerevisiae (31.0 %), *Pichia kudriavzevii* (13.8 %), *Clavispora lusitaniae* (8.6 %) and *Candida tropicalis* (5.2 %) were the yeast.

These results are similar with the findings of Lei & Jakobsen (2004) who identified *L. fermentum, W. confusa, P. acidilactici, P. pentosaceus, L. paraplantarum* and *L. salivarius* in *Hausa koko* fermentation based on sequencing of the 16S rRNA gene. They did not identify the yeast population. In the present work, a lot more LAB species were encountered including *L. pontis*, *L. reuteri, L. paracasei* and *S. harbinensis.* In addition, many LAB species were identified at each processing stage than was reported previously. In the present study, the yeast population during *Hausa koko* production was dominated by *S. paradoxus* and *S. cerevisiae* accounting for about 70 % of the total yeast population. *S. cerevisiae* has been reported extensively in fermented foods (Kigigha et al., 2016; Achi & Ukwuru, 2015). *Saccharomyces paradoxus* on the other hand has only been reported in a few instances in cereals using molecular characterisation involving sequencing (Obinna-Echem et al., 2014; Naumova et al., 2003). *S. paradoxus* is the undomesticated relative of *S. cerevisiae*, they co-exist in a similar environment and exhibit indistinctive characteristics (Kowallik et al., 2015; Sniegowski et al., 2002). It is therefore likely that *S. cerevisiae* has been reported in some cases that molecular characterisation was not applied. All the other yeast have been associated with cereal fermented foods (Greppi et al., 2013; Pedersen et al., 2012). The LAB may contribute to the production of metabolites like organic acids, bacteriocins and many others to inhibit the growth of pathogenic and spoilage organisms to improve microbial safety of *Hausa koko* (Schnurer & Magnusson, 2005; O'Sullivan et al., 2002). Yeast also plays a key role in the production of ethanol, extracellular enzyme and flavour compounds (Omemu et al., 2007; Amoa-Awua et al., 2007).

Pre-screening of 90 identified LAB isolates using genome mining tools BAGEL 4 for predicting bacteriocin genes and Pathosystems Resource Integration Center (PATRIC) for predicting beneficial genomic features resulted in the selection of 27 isolates. These were species of *L. pontis* (16 isolates), *L. reuteri* (5), *L. fermentum* (4), *P. acidilactici* (1) and *P. pentosaceus* (1). All the *L. pontis*, *L. fermentum* and *L. reuteri* isolates showed predictive enterolysin A structural protein. The *P. pentosaceus* genome showed Bovicin 255, Penocin A and immunity structural protein, whilst *P. acidilactici* genome indicated putative encoding of Mersacidin and Enterolysin A variant structural proteins. These predicted bacteriocins have inhibitory activity against pathogens and other indicator organisms (Jiang et al., 2021; McAllister et al., 2011; Sass et al., 2008). Similar outcome was reported from the genome of *L. fermentum* isolates from selected cereal fermented foods from Nigeria using BAGEL 3 database and BLASTP (Abdulkarim et al., 2020). In this study, the isolates were also predicted to have genomic features including the absence of antimicrobial resistance (AMR) genes, presence of genes related to nutritive and enzymatic compound production including riboflavin, folate, niacin, thiamine and others.

These 27 isolates were therefore selected and tested further (*in vitro*) for technological and probiotic properties. They showed good rates of acidification which is necessary to produce organic acids quickly to reduce the pH, inhibit microbial contaminants, improve sensory attributes and likely reduce the duration for the fermentation of millet (Annan et al., 2015; Min et al., 2007). They showed strong inhibitory activity against foodborne indicator organisms *Salmonella enterica sv typhymurium* Lt2, *Bacillus cereus* VLAG 699, *Enterococcus faecium* ATCC 6057, *Staphylococcus aureus* FI10739, *Enterococcus faecalis* FI9187 and *E. coli* RMEC0157 NCCBI 100282. *Micrococcus luteus* FI10640 was the least susceptible to the isolates. This is an indication of the isolates' ability to control the growth of pathogens and indicator organisms to improve the

safety of *Hausa koko* (Rattanachaikunsopon & Phumkhachorn, 2010). About half of the LAB isolates produced very little or no amylase enzyme, whilst the other half showed substantial amylase activity which is needed for saccharification of starch to yield fermentable sugars (Egwim & Oloyede, 2006) and in releasing of nutrients (Oguntoyinbo & Narbad, 2012). Low production of exopolysaccharides (EPS) was also observed. EPS is key in improving the textural and sensory properties of fermented products (Owusu-Kwarteng et al., 2015). These isolates exhibited good tolerance and survival in acid conditions at low to neutral pH (2.5, 3.5, 4.5, 6.0 and 7.0) even though some strains grew partially or did not grow at all at pH 2.5. Although none grew in pH 1.5, they still demonstrated some ability to withstand the organic acid conditions from their metabolism in the fermented millet matrix and survival in normal gastrointestinal pH in the stomach (Owusu-Kwarteng et al., 2015; Psomas et al., 2001). They also exhibited tolerance and survival against the antimicrobial agent, bile, present in the intestinal tract at different concentrations (0.3, 0.5 and 1.0 %). Similarly, except for *Clavispora lusitaniae*, a known non-beneficial yeast, probiotic potentials determined for 53 out of 58 yeast isolates exhibited good attributes. Their tolerance to low to neutral pH conditions (2.0, 3.0, 5.5 and 7.0), bile (0.3, 0.5 and 1.0 %), high temperatures at 37 °C, salt conditions (4 and 6 %) was a good indication of survival throughout the human gastrointestinal tract (GIT) according to Pedersen et al., (2012).

Out of these, three potential probiotic strains of LAB isolates *L. reuteri* LDOD-Sud, *L. pontis* LTAD-12g and *L. fermentum* LMAN-Sdb, and two yeast isolates, *S. cerevisiae* YSUN-Sud and *P. kudriavzevii* YTAD-12j were selected for further studies in the development of a starter culture or inoculum enrichment during millet fermentation. This is because the results of the *in-vitro* test strongly suggested that these strains are potential probiotics due to the expression of some key technological and probiotic attributes. They were paired in 15 double and triple combinations for

starter culture trials, where practically all of them demonstrated good growth with high viable cell populations and rapid acidification rate when inoculated into millet slurry, either as pure cultures or as inoculum enrichment. An attribute a starter culture must possess and has been reported in other studies (De Melo Pereira et al., 2020 & 2018; Houngbédji et al., 2018). The 15 combinations were also used to ferment (12 h) aflatoxins B1, B2 and G2 infected millet slurries to test their aflatoxin inhibiting properties. The extent of reduction varied but most of them were able to reduce the levels of aflatoxins during the fermentation period. The reduction or even total inhibition may be attributed to a decrease in pH, interactions between the LAB-yeast and the mycotoxins present, the ability of LAB to trap some mycotoxins, interactions between the microbial populations and inhibiting compounds produced by LAB (Dalié et al., 2010; Gerez et al., 2009; Wu et al., 2009; Shetty & Jespersen, 2006).

Despite how well these starter culture combinations performed in terms of technological and probiotic potentials, their acceptability by consumers is very important. For that reason, all the combinations were used for millet porridge production and their acceptability was evaluated. They were judged on their aroma, colour, consistency, taste and overall acceptability. The millet porridge was prepared using both irradiated (5 kGy) and non-irradiated millet slurries without spices to prevent the masking of the starter culture attributes by the spices and evaluated by a panel of 20 members. From both evaluations, combinations RFC, RFK, RK, RF and FC which are associated with most cereal fermented foods in Ghana like *kenkey*, *koko*, *fura* and others (Annan et al., 2015; Owusu-Kwarteng et al., 2012; Ackaah-Gyasi, 2010) were preferred. They were then used as inoculum enrichment for the fermentation of millet for *Hausa koko* production following the traditional process with spices added and some process modification. They were evaluated again for acceptability by the same 20 panelists. The most preferred starter culture fermented

Hausa koko was RFC, that is, *Limosilactobacillus reuteri* LDOD-Sud (R) + *Limosilactobacillus fermentum* LMAN-Sdb (F) + *Saccharomyces cerevisiae* YSUN-Sud (C) combination. It was rated highest in terms of aroma, consistency and overall acceptability.

The performance of the most preferred starter culture, simply referred to as RFC *Hausa koko* Starter Culture (RFCH), was evaluated (*in situ*) in the fermentation of pearl millet in two ways. The first was done during the fermentation of millet slurries involving Starter RFCH as either pure starter culture or inoculum enrichment on a pilot scale. The second was done during the fermentation of millet dough involving Starter RFCH as an inoculum enrichment at a semi-industrial scale production of *Hausa koko* flour. The use of Starter RFCH as inoculum enrichment recorded a significantly lower pH in 12 h of fermentation with corresponding higher TTA values in comparison to its use as pure starter culture during the pilot study. This could be explained by a higher population of LAB and yeasts in the inoculum enrichment, since the natural microbiota of the millet was present in inoculum enrichment leading to a higher population of fermenting organisms in the inoculum enrichment fermentation with carbohydrate degradation for acidification (Onuoha et al., 2017; Wakil & Kazeem, 2012). As a result, a faster microbial load reduction or elimination of potential pathogens and indicator organisms including Enterobacteriaceae, *E. coli*, *S. aureus* and *B. cereus* during both pilot fermentations was observed. Faster acidification and antimicrobial compounds production increases the shelf life of the product and improves its microbial safety as expected of a starter culture (De Melo Pereira et al., 2018; Soro-Yao et al., 2014). Significant increases were observed in moisture, protein, iron, calcium and phosphorus levels during pilot scale fermentation of the millet slurries, whilst carbohydrate, fat and energy decreased as reported in similar studies (Onuoha et al., 2017; Simwaka et al., 2017; Babalola & Giwa, 2012). These effects were greater in the fermentations in which starter RFCH

was used either as pure starter culture or as inoculum enrichment, in comparison to the spontaneous fermentation (control). The inoculum enriched fermentation showed the highest increases of these constituents, which may be due to a higher microbial population since these two fermentations were inoculated with the same concentrations of starter RFCH. Although not under a strictly controlled fermentation set up, RFCH starter culture demonstrated faster acidification and stability, which is a suitability criterion for its usage for large-scale production. It was also able to dominate the indigenous microbial population of the fermenting matrix as expected. Together with other desirable traits, RFCH starter culture was able to improve the quality of the fermented dough and reduced the fermentation time from the normal 48 - 72 h to only 12 h during semi-industrial scale fermentation. RFCH starter culture inoculum enriched 12 h fermented dough flour and the spontaneously fermented dough flour were prepared into porridge and assessed for consumer acceptability by the 20-member panelist. They adjudged the *Hausa koko* produced from starter RFCH fermented millet dough highest in terms of aroma, taste and overall acceptability as compared to the spontaneously fermented sample. Appearance and consistency even though improved they were not significantly different ($P \leq 0.05$).

8.2 Conclusions

The study presents the most comprehensive bacterial profile yet reported at all processing stages of *Hausa koko*. In total, over 400 bacterial types were profiled and 33 metabolites quantified at all the different processing stages of *Hausa koko* production. The metabolites included alcohols, sugars, amino acids and some other key compounds using NMR spectroscopy.

Using whole genome sequencing (WGS) and Sanger sequencing methods, 9 dominant lactic acid bacteria (LAB) isolates and 5 yeast isolates involved in *Hausa koko* fermentation process were characterised. *Limosilactobacillus pontis, Pediococcus acidilactici, Limosilactobacillus fermentum, Pediococcus pentosaceus, Limosilactobacillus reuteri, Weissella confusa, Saccharomyces cf. cerevisiae/paradoxus, Saccharomyces cerevisiae* and *Pichia kudriavzevii* are the predominant LAB and yeast associated with *Hausa koko* production. *Schleiferilactobacillus harbinensis, Lactiplantibacillus plantarum, Lacticaseibacillus paracasei, Clavispora lusitaniae* and *Candida tropicalis* also occurred in low populations. This is the first time *L. pontis* and *S. harbinensis* have been reported for fermented cereal/millet/*Hausa koko* in Ghana, whilst limited reports exist on *S. cf. cerevisiae/paradoxus* in Ghanaian fermented foods.

Most of the dominant LAB and yeast strains exhibited good technological and probiotic characteristics which included fast growth rate, good acidification rate, antimicrobial activity, bile and pH tolerance.

Fifteen (15) LAB and yeast combinations were tested and out of these, a combination of 2 LAB strains (*Limosilactobacillus reuteri* LDOD-Sud, *Limosilactobacillus fermentum* LMAN-Sdb) and 1 yeast strain (*Saccharomyces cerevisiae* YSUN-Sud) exhibited the highest potential based on their performance and were used to develop RFCH starter culture.

RFCH starter culture which exhibited the highest potential also exhibited better quality and safety characteristics compared to the spontaneous fermentation in *Hausa koko* production. RFCH starter culture may therefore be considered for control fermentation and as inoculum in indigenous population.

8.3 Recommendations

1. Characterisation of the predicted genes responsible for bacteriocin, nutrients and enzymes production, as well as those responsible for aflatoxin reduction in millet fermentation, should be further investigated using the LAB whole genome sequences. Other functional properties of the LAB isolates should also be investigated using genome mining tools.

2. Metabolomic studies on *Hausa koko* fermented with RFCH stater culture should be conducted to identify the flavor and aromatic compounds accounting for the enhanced sensory attributes.

3. Additional investigations into RFC *Hausa koko* starter culture's ability to survive gut transit when ingested should be conducted. In addition to a whole gut microbiome study in both healthy and disease states in humans, utilising *in vitro* models of the colon using new methods in next generation sequencing should be conducted.

4. Suitable substrate, storage condition, shelf life and packaging for dehydrated RFC *Hausa koko* starter culture should be investigated and made readily accessible in a sachet form as they are more stable and much easier to handle. In that form, commercial processors following a simple Standard Operating Procedure can easily use them. This will offer excellent possibilities for greater control over the fermentation process.

REFERENCES

Abate, A.R., Hung, T., Sperling, R.A., Mary, P., Rotem, A., Agresti, J.J., Weiner, M.A. & Weitz, D.A. (2013). DNA sequence analysis with droplet-based microfluidics. *Lab on a Chip*, *13*(24), 4864-4869.

Abbas, C. A. (2006). Production of antioxidants, aromas, colours, flavours, and vitamins by yeasts. In *Yeasts in food and beverages* (pp. 285-334). Springer, Berlin, Heidelberg.

Abdalla, I. G., Ahmed, K. E., Abdelbagi, A. O., & Babiker, E. E. (2015). Effect of radiation and/or traditional processings on antinutrients and HCl extractability of calcium, phosphorus and iron of sorghum cultivars. *Journal of Food Science and Technology*, *52*(3), 1705-1711.

AbdElatif, S., Elsayed, M., Bahout, A., & Bayoumi, M. (2016). Studies on beneficial yeasts isolated from some Egyptian dairy products. *Zagazig Veterinary Journal*, *44*(1), 75-84.

Abdulkarim, I. H., Mohammed, S. S. D., & Orukotan, A. A. (2020). Gene Identification for Bacteriocin Production by Lactic Acid Bacteria Isolated from Selected Fermented Foods. *Asian Journal of Biochemistry, Genetics and Molecular Biology*, 1-12.

Abegaz, K. (2007). Isolation, characterisation and identification of lactic acid bacteria involved in traditional fermentation of borde, an Ethiopian cereal beverage. *African Journal of Biotechnology*, *6*(12), 1469-1478.

Abriouel, H., Omar, N. B., López, R. L., Martínez-Cañamero, M., Keleke, S., & Gálvez, A. (2006). Culture-independent analysis of the microbial composition of the African traditional fermented foods poto poto and dégué by using three different DNA extraction methods. *International Journal of Food Microbiology*, *111*(3), 228-233.

Achi, O. K. (2005). The potential for upgrading traditional fermented foods through biotechnology. *African Journal of Biotechnology*, *4*(5), 375-380.

Achi, O. K., & Ukwuru, M. (2015). Cereal-based fermented foods of Africa as functional foods. *International Journal of Microbiology and Application*, *2*(4), 71-83.

Ackaah-Gyasi, N. A. (2010). Predominant microorganisms associated with fermentation of millet into maasa. Mphil. Thesis Submitted to University of Ghana.

Adebiyi, J. A., Obadina, A. O., Adebo, O. A., & Kayitesi, E. (2017). Comparison of nutritional quality and sensory acceptability of biscuits obtained from native, fermented, and malted pearl millet (*Pennisetum glaucum*) flour. *Food Chemistry*, *232*, 210-217.

Adebo, O. A., Njobeh, P. B., Adebiyi, J. A., Gbashi, S., & Kayitesi, E. (2017). Food metabolomics: a new frontier in food analysis and its application to understanding fermented foods. *Functional Food-Improve Health through Adequate Food, IntechOpen.*

Adebo, O.A., Oyeyinka, S.A., Adebiyi, J.A., Feng, X., Wilkin, J.D., Kewuyemi, Y.O., Abrahams, A.M. & Tugizimana, F. (2021). Application of gas chromatography–mass spectrometry (GC-MS)-based metabolomics for the study of fermented cereal and legume foods: A review. *International Journal of Food Science & Technology*, *56*(4), 1514-1534.

Adekunle, A.A., Ellis-Jones, J., Ajibefun, I., Nyikal, R.A., Bangali, S., Fatunbi, A.O. & Angé, A. (2013, June). Agricultural innovation in sub-Saharan Africa: Experiences from multiple stakeholder approaches. Accra, Ghana: Forum for Agricultural Research in Africa (FARA).

Adesokan, I. A., Ekanola, Y. A., & Okanlawon, B. M. (2010). Influence of cultural conditions on hydrogen peroxide production by lactic acid bacteria isolated from some Nigerian traditional fermented foods. *African Journal of Microbiology Research*, *4*(19), 991-1996.

Adesulu-Dahunsi, A. T., Jeyaram, K., & Sanni, A. I. (2018). Probiotic and technological properties of exopolysaccharide producing lactic acid bacteria isolated from cereal-based nigerian fermented food products. *Food Control*, *92*, 225-231.

Adiandri, R. S., & Hidayah, N. (2019, September). Effect of Fermentation using Lactobacillus casei on the Physicochemical and Functional Properties of Sorghum Flour. In *IOP Conference Series: Earth and Environmental Science* (Vol. 309, No. 1, p. 012025). IOP Publishing.

Adimpong, D. B., Nielsen, D. S., Sørensen, K. I., Derkx, P. M., & Jespersen, L. (2012). Genotypic characterisation and safety assessment of lactic acid bacteria from indigenous African fermented food products. *BMC Microbiology*, *12*(1), 1-12.

Agarry, O. O., Nkama, I., & Akoma, O. (2010). Production of Kunun-zaki (A Nigerian fermented cereal beverage) using starter culture. *International Research Journal of Microbiology*, *1*(2), 18-25.

Agostini, C., Eckert, C., Vincenzi, A., Machado, B.L., Jordon, B.C., Kipper, J.P., Dullius, A., Dullius, C.H., Lehn, D.N., Sperotto, R.A. & Pozzobon, A (2018). Characterisation of technological and probiotic properties of indigenous *Lactobacillus* spp. from south Brazil. *3 Biotech*, *8*(11), 1-12.

Ahene, R. E., Odamtten, G. T., & Owusu, E. (2011). Fungal and bacterial contaminants of six spices and spice products in Ghana. *African Journal of Environmental Science and Technology*, *5*(9), 633-640.

Ahlberg, S. H., Joutsjoki, V., & Korhonen, H. J. (2015). Potential of lactic acid bacteria in aflatoxin risk mitigation. *International journal of food microbiology*, *207*, 87-102.

Aidoo, K. E., & Nout, M. R. (2010). Functional yeasts and molds in fermented foods and beverages. In *Fermented foods and beverages of the world* (pp. 139-160). CRC Press.

Aka, S., Konan, G., Fokou, G., Dje, K. M., & Bonfoh, B. (2014). Review on African traditional cereal beverages. *Am. J. Res. Commun*, *2*(5), 103-153.

Akabanda, F., Owusu-Kwarteng, J., Tano-Debrah, K., Parkouda, C., & Jespersen, L. (2014). The use of lactic acid bacteria starter culture in the production of Nunu, a spontaneously fermented milk product in Ghana. *International Journal of Food Science*, *2014*.

Akayeti, E. (2019). Research Work on Naara (Early) and Zea (Late) Millets in Ghana. *Modern Ghana, Science and Environment*. https://www.modernghana.com/news/954914/research-work-on-naara-ealy-and-zea-late-mill.html.

Akpinar-Bayizit, A., Yilmaz-Ersan, L., & Ozcan, T. (2010). Determination of boza's organic acid composition as it is affected by raw material and fermentation. *International Journal of Food Properties*, *13*(3), 648-656.

Alfonzo, A., Ventimiglia, G., Corona, O., Di Gerlando, R., Gaglio, R., Francesca, N., Moschetti, G. & Settanni, L. (2013). Diversity and technological potential of lactic acid bacteria of wheat flours. *Food Microbiology*, *36*(2), 343-354.

Ali, A. A., & Mustafa, M. M. (2009). Use of starter cultures of lactic acid bacteria and yeasts in the preparation of kisra, a Sudanese fermented food. *Pakistan Journal of Nutrition*, *8*(9), 1349-1353.

Ali, M. A., El Tinay, A. H., & Abdalla, A. H. (2003). Effect of fermentation on the in vitro protein digestibility of pearl millet. *Food Chemistry*, *80*(1), 51-54.

Allen, S.J., Jordan, S., Storey, M., Thornton, C.A., Gravenor, M.B., Garaiova, I., Plummer, S.F., Wang, D. & Morgan, G. (2014). Probiotics in the prevention of eczema: a randomised controlled trial. *Archives of Disease in Childhood*, *99*(11), 1014-1019.

Almeida, E. G., Rachid, C. C., & Schwan, R. F. (2007). Microbial population present in fermented beverage 'cauim'produced by Brazilian Amerindians. *International Journal of Food Microbiology*, *120*(1-2), 146-151.

Amadou, I., Gbadamosi, O. S., & Le, G. W. (2011). Millet-based traditional processed foods and beverages—A review. *Cereal Foods World*, *56*(3), 115-121.

Amadou, I., Gounga, M. E., & Le, G. W. (2013). Millets: Nutritional composition, some health benefits and processing-A review. *Emirates Journal of Food and Agriculture*, 501-508.

Amaike, S., & Keller, N. P. (2011). *Aspergillus flavus*. *Annual Review of Phytopathology*, *49*, 107-133.

Amankona, C. (2016). *Development of Starter Culture for Fermentation of Millet into Fura and Preservation of Fura by Gamma Radiation* (MPhil dissertation, University of Ghana).

Amankwah, E. A., Barimah, J., Acheampong, R., Addai, L. O., & Nnaji, C. O. (2009). Effect of fermentation and malting on the viscosity of maize-soyabean weaning blends. *Pakistan Journal of Nutrition*, *8*(10), 1671-1675.

Ammor, M. S., & Mayo, B. (2007). Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. *Meat Science*, *76*(1), 138-146.

Ammor, S., Tauveron, G., Dufour, E., & Chevallier, I. (2006). Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility: 1—Screening and characterisation of the antibacterial compounds. *Food Control*, *17*(6), 454-461.

Amoa-Awua, W. K. A., Appoh, F. E., & Jakobsen, M. (1996). Lactic acid fermentation of cassava dough into agbelima. *International Journal of Food Microbiology*, *31*(1-3), 87-98.

Amoa-Awua, W. K., Frisvad, J. C., Sefa-Dedeh, S., & Jakobsen, M. (1997). The contribution of moulds and yeasts to the fermentation of 'agbelima'cassava dough. *Journal of Applied Microbiology*, *83*(3), 288-296.

Amoa-Awua, W. K., Sampson, E., & Tano-Debrah, K. (2007). Growth of yeasts, lactic and acetic acid bacteria in palm wine during tapping and fermentation from felled oil palm (Elaeis guineensis) in Ghana. *Journal of Applied Microbiology*, *102*(2), 599-606.

Amoo-Gyasi, M. (2013). The microbial quality of a traditional fermented cow milk product "burkina" sold in the Madina market in the Greater Accra region of Ghana. BTech. Thesis Submitted to Accra Technical University.

Anal, A. K. (2019). Quality ingredients and safety concerns for traditional fermented foods and beverages from Asia: A review. *Fermentation*, *5*(1), 8.

Annan, T., Obodai, M., Anyebuno, G., Tano-Debrah, K., & Amoa-Awua, W. K. (2015). Characterisation of the dominant microorganisms responsible for the fermentation of dehulled maize grains into nsiho in Ghana. *African Journal of Biotechnology*, *14*(19), 1640-1648.

Antony, U., & Chandra, T. S. (1999). Enzymatic treatment and use of starters for the nutrient enhancement in fermented flour of red and white varieties of finger millet (*Eleusine coracana*). *Journal of Agricultural and Food Chemistry*, *47*(5), 2016-2019.

Anukam, K. C., & Reid, G. (2009). African traditional fermented foods and probiotics. *Journal of Medicinal Food*, *12*(6), 1177-1184.

Apena, A., Opeolu, S. O., Bamidele, F. A., & Shittu, A. (2015). Nutrient changes during fermentation of some selected cereals. *Sky Journal of Biochemistry Research*, *4*(2), 010-012.

Arena, M. P., Silvain, A., Normanno, G., Grieco, F., Drider, D., Spano, G., & Fiocco, D. (2016). Use of Lactobacillus plantarum strains as a bio-control strategy against food-borne pathogenic microorganisms. *Frontiers in Microbiology*, *7*, 464.

Association of Official Analytical Chemists (AOAC). (2005). Official Methods of Analysis Method No. 3.4.11.

Association of Official Analytical Chemists (AOAC). (2005). Official Methods of Analysis Method No. 4.8.03.

Association of Official Analytical Chemists (AOAC). (2016). Official Methods of Analysis 20th Ed, Method No 32.1.03. International, Washington DC, USA.

Association of Official Analytical Chemists (AOAC). (2016). Official Methods of Analysis 20th Ed, Method No. 32.1.05. International, Washington DC, USA.

Association of Official Analytical Chemists (AOAC). (2016). Official Methods of Analysis 20th Ed, method No. 4.2.09. International, Washington DC, USA.

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

Attaie, R. (2009). Quantification of volatile compounds in goat milk Jack cheese using static headspace gas chromatography. *Journal of Dairy Science*, *92*(6), 2435-2443.

Atter, A. (2012). Microbial and chemical processes associated with burukutu, a Ghanaian fermented alcoholic beverage. M.Phil Thesis, Kwame Nkumah University of Science and Technology.

Atter, A., Diaz, M., Tano-Debrah, K., Kunadu, A.P.H., Mayer, M.J., Colquhoun, I.J., Nielsen, D.S., Baker, D., Narbad, A. & Amoa-Awua, W. (2021). Microbial Diversity and Metabolite Profile of Fermenting Millet in the Production of Hausa koko, a Ghanaian Fermented Cereal Porridge. *Frontiers in Microbiology*, 1752.

Atter, A., Obiri-Danso, K., & Amoa-Awua, W. K. (2014). Microbiological and chemical processes associated with the production of burukutu a traditional beer in Ghana. *International Food Research Journal*, *21*(5), 1769.

Aureli, P., Capurso, L., Castellazzi, A.M., Clerici, M., Giovannini, M., Morelli, L., Poli, A., Pregliasco, F., Salvini, F. & Zuccotti, G.V. (2011). Probiotics and health: an evidence-based review. *Pharmacological Research*, *63*(5), 366-376.

Axel, C., Röcker, B., Brosnan, B., Zannini, E., Furey, A., Coffey, A., & Arendt, E. K. (2015). Application of Lactobacillus amylovorus DSM19280 in gluten-free sourdough bread to improve the microbial shelf life. *Food Microbiology*, *47*, 36-44.

Axelsson, L. (2004). Lactic acid bacteria: classification and physiology. *Food science and technology-New York-Marcel Dekker-*, *139*, 1-66.

Aziz, N. H., Souzan, R. M., & Azza, A. S. (2006). Effect of γ-irradiation on the occurrence of pathogenic microorganisms and nutritive value of four principal cereal grains. *Applied Radiation and Isotopes*, *64*(12), 1555-1562.

Azizi, M. M. F., Ismail, S. I., Ina-Salwany, M. Y., Hata, E. M., & Zulperi, D. (2020). The emergence of Pantoea species as a future threat to global rice production. *Journal of Plant Protection Research*, 327-335.

Babalola, R. O., & Giwa, O. E. (2012). Effect of fermentation on nutritional and anti-nutritional properties of fermenting Soy beans and the antagonistic effect of the fermenting organism on selected pathogens. *International Research Journal of Microbiology*, *3*(10), 333-338.

Badau, M. H. (2006). Microorganisms associated with pearl millet cultivars at various malting stages. *Internet J. Food Safe*, *8*, 66-72.

Bahmani, M., Shirzad, H., Mirhosseini, M., Mesripour, A., & Rafieian-Kopaei, M. (2016). A review on ethnobotanical and therapeutic uses of fenugreek (Trigonella foenum-graceum L). *Journal of evidence-based complementary & alternative medicine*, *21*(1), 53-62.

Bakobie, N., Addae, A. S., Duwiejuah, A. B., Cobbina, S. J., & Miniyila, S. (2017). Microbial profile of common spices and spice blends used in Tamale, Ghana. *International Journal of Food Contamination*, *4*(1), 1-5.

Balli, D., Bellumori, M., Paoli, P., Pieraccini, G., Di Paola, M., De Filippo, C., Di Gioia, D., Mulinacci, N. & Innocenti, M. (2019). Study on a fermented whole wheat: Phenolic content, activity on PTP1B enzyme and in vitro prebiotic properties. *Molecules*, *24*(6), 1120.

Bamforth, C. W. (2005). Food, fermentation and Microorganisms. *Blackwell Publishing Ltd.* 31-60.

Bancalari, E., Castellone, V., Bottari, B., & Gatti, M. (2020). Wild *Lactobacillus casei* Group Strains: Potentiality to ferment plant derived juices. *Foods*, *9*(3), 314.

Barba, M., Czosnek, H., & Hadidi, A. (2014). Historical perspective, development and applications of next-generation sequencing in plant virology. *Viruses*, *6*(1), 106-136.

Barros, R. R., Carvalho, M. D. G. S., Peralta, J. M., Facklam, R. R., & Teixeira, L. M. (2001). Phenotypic and genotypic characterisation of Pediococcus strains isolated from human clinical sources. *Journal of Clinical Microbiology*, *39*(4), 1241-1246.

Bartowsky, E. J., & Henschke, P. A., (2004). The 'buttery'attribute of wine—diacetyl—desirability, spoilage and beyond. *International Journal of Food Microbiology*, *96*(3), 235-252.

Basch, E., Ulbricht, C., Kuo, G., Szapary, P., & Smith, M. (2003). Therapeutic applications of Fenugreek. (Fenugreek). *Alternative Medicine Review*, *8*(1), 20-28.

Bashir, K., Swer, T. L., Prakash, K. S., & Aggarwal, M. (2017). Physico-chemical and functional properties of gamma irradiated whole wheat flour and starch. *LWT-Food Science and Technology*, *76*, 131-139.

Bassi, D., Puglisi, E., & Cocconcelli, P. S. (2015). Comparing natural and selected starter cultures in meat and cheese fermentations. *Current Opinion in Food Science*, *2*, 118-122.

Batish, V. K., Roy, U., Lal, R., & Grower, S. (1997). Antifungal attributes of lactic acid bacteria—a review. *Critical Reviews in Biotechnology*, *17*(3), 209-225.

Behare. P. V., Singh, R., Kumar, M., Prajapati, J. B., & Singh, R. P. (2009). Exopolysaccharides of lactic acid bacteria: a review. *Journal of Food Science and Technology*, *46*(1), 1-11.

Bennett-Lartey, S. O., & Oteng-Yeboah, A. A. (2008). *Ghana country report on the state of plant genetic resources for food and agriculture*.

Bevilacqua, A., Corbo, M. R., & Sinigaglia, M. (2012). Selection of yeasts as starter cultures for table olives: a step-by-step procedure. *Frontiers in Microbiology*, *3*, 194.

Bibek, R. (2004). Health benefits of beneficial bacteria. *Fundamental food microbiology*, (Ed. 3), CRC Press, Boca Raton London New York Washington.

Birkenhauer, J. M., & Oliver, J. D. (2003). Use of diacetyl to reduce the load of Vibrio vulnificus in the eastern oyster, Crassostrea virginica. *Journal of Food Protection*, *66*(1), 38-43.

Blandino, A., Al-Aseeri, M. E., Pandiella, S. S., Cantero, D., & Webb, C. (2003). Cereal-based fermented foods and beverages. *Food Research International*, *36*(6), 527-543.

Bodaszewska-Lubas, M., Brzychczy-Włoch, M., Gosiewski, T., & Heczko, P. (2012). Antibacterial activity of selected standard strains of lactic acid bacteria producing bacteriocins: pilot study. *Postępy Higieny i Medycyny Doświadczalnej*, *66*.

Bogaerts, B., Nouws, S., Verhaegen, B., Denayer, S., Van Braekel, J., Winand, R., Fu, Q., Crombé, F., Piérard, D., Marchal, K. & Roosens, N.H. (2021). Validation strategy of a bioinformatics whole genome sequencing workflow for Shiga toxin-producing Escherichia coli using a reference collection extensively characterised with conventional methods. *Microbial Genomics*, *7*(3).

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J.C., Gerds, M.L., Hammes, W.P., Harnett, J., Huys, G., Laulund, S., Ouwehand, A. & Powell, I.B. (2012). Food fermentations: microorganisms with technological beneficial use. *International Journal of Food Microbiology*, *154*(3), 87-97.

Bourrie, B. C., Willing, B. P., & Cotter, P. D. (2016). The microbiota and health promoting characteristics of the fermented beverage kefir. *Frontiers in Microbiology*, *7*, 647.

Bowdish, D. M., Davidson, D. J., & Hancock, R. (2005). A re-evaluation of the role of host defence peptides in mammalian immunity. *Current Protein and Peptide Science*, *6*(1), 35-51.

Božanić, R., Tratnik, L. J., & Hruškar, M. (2003). Influence of culture activity on aroma components in yoghurts produced from goat's and cow's milk. *Acta Alimentaria*, *32*(2), 151-160.

Brandt, M. J. (2014). Starter cultures for cereal based foods. *Food Microbiology*, *37*, 41-43.

Bruce, S. J., Guy, P. A., Rezzi, S., & Ross, A. B. (2010). Quantitative measurement of betaine and free choline in plasma, cereals and cereal products by isotope dilution LC-MS/MS. *Journal of Agricultural and Food Chemistry*, *58*(4), 2055-2061.

Buckenhüskes, H. J. (1993). Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. *FEMS Microbiology Reviews*, *12*(1-3), 253-271.

Caggianiello, G., Kleerebezem, M., & Spano, G. (2016). Exopolysaccharides produced by lactic acid bacteria: from health-promoting benefits to stress tolerance mechanisms. *Applied Microbiology and Biotechnology*, *100*(9), 3877-3886.

Campbell-Platt, G. (1987). *Fermented foods of the world. A dictionary and guide*. Butterworths.

Cao, Y., Fanning, S., Proos, S., Jordan, K., & Srikumar, S. (2017). A review on the applications of next generation sequencing technologies as applied to food-related microbiome studies. *Frontiers in Microbiology*, *8*, 1829.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I. & Huttley, G.A. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), 335-336.

Carafa, I., Nardin, T., Larcher, R., Viola, R., Tuohy, K., & Franciosi, E. (2015). Identification and characterisation of wild lactobacilli and pediococci from spontaneously fermented Mountain cheese. *Food Microbiology*, *48*, 123-132.

Cerning, J. (1994). Polysaccharides exocellulaires produits par les bactéries lactiques. In Bactéries Lactiques. Aspects Fondamentaux et Technologiques., 1, 309–329

Chakravorty, S., Helb, D., Burday, M., Connell, N., & Alland, D. (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *Journal of Microbiological Methods*, *69*(2), 330-339.

Champagne, C. P., Tompkins, T. A., Buckley, N. D., & Green-Johnson, J. M. (2010). Effect of fermentation by pure and mixed cultures of *Streptococcus thermophilus* and *Lactobacillus helveticus* on isoflavone and B-vitamin content of a fermented soy beverage. *Food Microbiology*, *27*(7), 968-972.

Charalampopoulos, D., Wang, R., Pandiella, S. S., & Webb, C. (2002). Application of cereals and cereal components in functional foods: a review. *International Journal of Food Microbiology*, *79*(1-2), 131-141.

Chaudhary, N., Sharma, A. K., Agarwal, P., Gupta, A., & Sharma, V. K. (2015). 16S classifier: a tool for fast and accurate taxonomic classification of 16S rRNA hypervariable regions in metagenomic datasets. *PloS one*, *10*(2), e0116106.

Chen, G., Chen, C., & Lei, Z. (2017). Meta-omics insights in the microbial community profiling and functional characterisation of fermented foods. *Trends in Food Science & Technology*, *65*, 23-31.

Chen, Y., Liu, W., Xue, J., Yang, J., Chen, X., Shao, Y., Kwok, L.Y., Bilige, M., Mang, L. & Zhang, H. (2014). Angiotensin-converting enzyme inhibitory activity of Lactobacillus helveticus strains from traditional fermented dairy foods and antihypertensive effect of fermented milk of strain H9. *Journal of Dairy Science*, *97*(11), 6680-6692.

Cifuentes, A. (2009). Food analysis and foodomics. *Journal of Chromatography A*, *43*(1216), 7109.

Claesson, M. J., Wang, Q., O'Sullivan, O., Greene-Diniz, R., Cole, J. R., Ross, R. P., & O'Toole, P. W. (2010). Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. *Nucleic Acids Research*, *38*(22), 1-13.

Clark, S., & Winter, C. K. (2015). Diacetyl in foods: a review of safety and sensory characteristics. *Comprehensive Reviews in Food Science and Food Safety*, *14*(5), 634-643.

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International journal of food microbiology*, *71*(1), 1-20.

Cocolin, L., Dolci, P., & Rantsiou, K. (2011). Biodiversity and dynamics of meat fermentations: the contribution of molecular methods for a better comprehension of a complex ecosystem. *Meat Science*, *89*(3), 296-302.

Cogan, T. M., & Hill, C. (1993). Cheese starter cultures. In *Cheese: chemistry, physics and microbiology* (pp. 193-255). Springer, Boston, MA.

Collins, E. B., & Aramaki, K. (1980). Production of hydrogen peroxide by Lactobacillus acidophilus. *Journal of dairy science*, *63*(3), 353-357.

Collins, F. W., Mesa-Pereira, B., O'Connor, P. M., Rea, M. C., Hill, C., & Ross, R. P. (2018). Reincarnation of bacteriocins from the Lactobacillus pangenomic graveyard. *Frontiers in microbiology*, *9*, 1298.

Corsetti, A., & Settanni, L. (2007). Lactobacilli in sourdough fermentation. *Food Research International*, *40*(5), 539-558.

Corsetti, A., Gobbetti, M., Rossi, J., & Damiani, P. (1998). Antimould activity of sourdough lactic acid bacteria: identification of a mixture of organic acids produced by Lactobacillus sanfrancisco CB1. *Applied Microbiology and Biotechnology*, *50*(2), 253-256.

Cotter, P. D., Hill, C., & Ross, R. P. (2005). Bacteriocins: developing innate immunity for food. *Nature Reviews Microbiology*, *3*(10), 777-788.

Cotter, P. D., Ross, R. P., & Hill, C. (2013). Bacteriocins—a viable alternative to antibiotics?. *Nature Reviews Microbiology*, *11*(2), 95-105.

Cousin, M. A. (1994). Foodborne Mold Identification Workshop. Purdue University, West Lafayette, 1160.

Cselovszky, J., Wolf, G., & Hammes, W. P. (1992). Production of formate, acetate, and succinate by anaerobic fermentation of Lactobacillus pentosus in the presence of citrate. *Applied Microbiology and Biotechnology*, *37*(1), 94-97.

Dalié, D. K. D., Deschamps, A. M., & Richard-Forget, F. (2010). Lactic acid bacteria–Potential for control of mould growth and mycotoxins: A review. *Food Control*, *21*(4), 370-380.

Dalié, D., Pinson-Gadais, L., Atanasova-Penichon, V., Marchegay, G., Barreau, C., Deschamps, A., & Richard-Forget, F. (2012). Impact of Pediococcus pentosaceus strain L006 and its metabolites on fumonisin biosynthesis by Fusarium verticillioides. *Food Control*, *23*(2), 405-411.

Daniels, J. A., Krishnamurthi, R., & Rizvi, S. S. (1985). A review of effects of carbon dioxide on microbial growth and food quality. *Journal of Food Protection*, *48*(6), 532-537.

David, O. M., & Aderibigbe, E. Y. (2010). Microbiology and proximate composition of ogiri, a pastry produced from different melon seeds. *New York Science Journal*, *3*(4), 18-27.

Davidson, B. E., Llanos, R. M., Cancilla, M. R., Redman, N. C., & Hillier, A. J. (1995). Current research on the genetics of lactic acid production in lactic acid bacteria. *International Dairy Journal*, *5*(8), 763-784.

De Angelis, M., Rizzello, C.G., Fasano, A., Clemente, M.G., De Simone, C., Silano, M., De Vincenzi, M., Losito, I. & Gobbetti, M. (2006). VSL# 3 probiotic preparation has the capacity to hydrolyze gliadin polypeptides responsible for celiac sprue probiotics and gluten intolerance. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1762*(1), 80-93.

De Man, J. C., Rogosa, D., & Sharpe, M. E. (1960). A medium for the cultivation of *lactobacilli*. *Journal of Applied Bacteriology*, *23*(1), 130-135.

De Melo Pereira, G. V., de Oliveira Coelho, B., Júnior, A. I. M., Thomaz-Soccol, V., & Soccol, C. R. (2018). How to select a probiotic? A review and update of methods and criteria. *Biotechnology Advances*, *36*(8), 2060-2076.

De Melo Pereira, G., De Carvalho Neto, D. P., Junqueira, A. C. D. O., Karp, S. G., Letti, L. A., Magalhães Júnior, A. I., & Soccol, C. R. (2020). A review of selection criteria for starter culture development in the food fermentation industry. *Food Reviews International*, *36*(2), 135-167.

De Roos, J., & De Vuyst, L. (2018). Acetic acid bacteria in fermented foods and beverages. *Current Opinion in Biotechnology*, *49*, 115-119.

De Souza Motta, A., & Gomes, M. D. S. M. (2015). Technological and functional properties of lactic acid bacteria: the importance of these microorganisms for food. *Rev. do Inst. Laticinios Candido Tostes*, *70*, 172-184.

De Valdez, G. F., Gerez, C. L., Torino, M. I., & Rollán, G. (2010). New trends in cereal-based products using lactic acid bacteria. *Biotechnology of Lactic acid Bacteria: Novel Applications. Oxford, UK: Blackwell Publishing*, 273-287.

De Vuyst, L., & Leroy, F. (2007). Bacteriocins from lactic acid bacteria: production, purification, and food applications. *Journal of Molecular Microbiology and Biotechnology*, *13*(4), 194-199.

De Vuyst, L., & Neysens, P. (2005). The sourdough microflora: biodiversity and metabolic interactions. *Trends in Food Science & Technology*, *16*(1-3), 43-56.

De Vuyst, L., De Vin, F., Vaningelgem, F., & Degeest, B. (2001). Recent developments in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. *International Dairy Journal*, *11*(9), 687-707.

Di Stefano, E., White, J., Seney, S., Hekmat, S., McDowell, T., Sumarah, M., & Reid, G. (2017). A novel millet-based probiotic fermented food for the developing world. *Nutrients*, *9*(5), 529.

Diaz, M., Kellingray, L., Akinyemi, N., Adefiranye, O.O., Olaonipekun, A.B., Bayili, G.R., Ibezim, J., Du Plessis, A.S., Houngbédji, M., Kamya, D. & Mukisa, I.M. (2019). Comparison of the microbial composition of African fermented foods using amplicon sequencing. *Scientific Reports*, *9*(1), 1-8.

Diaz, M., Sayavedra, L., Atter, A., Mayer, M. J., Saha, S., Amoa-Awua, W., & Narbad, A. (2020). Lactobacillus garii sp. nov., isolated from a fermented cassava product. *International Journal of Systematic and Evolutionary Microbiology*, *70*(5), 3012.

Diener, U. L., Cole, R. J., Sanders, T. H., Payne, G. A., Lee, L. S., & Klich, M. A. (1987). Epidemiology of aflatoxin formation by Aspergillus flavus. *Annual Review of Phytopathology*, *25*(1), 249-270.

Dina, A. M., Ekbal, M. I., Adham, M. A., & Hamdi, A. M. (2013). Effect of Pediococcus acidilactici and its bacteriocin on soft cheese quality and validity. *Benha Veterinary Medical Journal*, *24*(1), 64-76.

Dinsdale, E.A., Edwards, R.A., Hall, D., Angly, F., Breitbart, M., Brulc, J.M., Furlan, M., Desnues, C., Haynes, M., Li, L. & McDaniel, L. (2008). Functional metagenomic profiling of nine biomes. *Nature*, *452*(7187), 629-632.

Diop, M. B., Dubois Dauphin, R., Tine, E., Ngom, A., Destain, J., & Thonart, P. (2007). Bacteriocin producers from traditional food products. *Biotechnologie, Agronomie, Société et Environnement*, *11*(4), 275-281.

Dobson, A., O'Sullivan, O., Cotter, P. D., Ross, P., & Hill, C. (2011). High-throughput sequence-based analysis of the bacterial composition of kefir and an associated kefir grain. *FEMS Microbiology Letters*, *320*(1), 56-62.

Douillard, F. P., & De Vos, W. M. (2014). Functional genomics of lactic acid bacteria: from food to health. *Microbial Cell Factories*, *13*(1), 1-21.

Dovlo, F. E. (1975). Maize in the Ghanaian diet. *The Home Scientist*, *2*(3), 13–29.

Draper, K., Ley, C., & Parsonnet, J. (2017). Probiotic guidelines and physician practice: a cross-sectional survey and overview of the literature. *Beneficial Microbes*, *8*(4), 507-519.

Durazzi, F., Sala, C., Castellani, G., Manfreda, G., Remondini, D., & De Cesare, A. (2021). Comparison between 16S rRNA and shotgun sequencing data for the taxonomic characterisation of the gut microbiota. *Scientific Reports*, *11*(1), 1-10.

Dziezak, J. D. (1990). Acidulants: ingredients that do more than meet the acid test. *Food Technology (Chicago)*, *44*(1), 76-83.

Edima-Nyah, A. P., Ntukidem, V. E., & James, E. I. (2019). Development and Quality Assessment of Breakfast Cereals from Blends of Whole Yellow Maize (Zea mays), Soybean (Glycine max) and Unripe Banana (Musa sapientum). *Asian Journal of Agriculture and Food Sciences*, *7*(4).

Egwim, E. C., & Oloyede, O. B. (2006). Comparison of a-amylase activity in some sprouting Nigerian cereals. *Biokemistri*, *18*(1).

Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., Peluso, P., Rank, D., Baybayan, P., Bettman, B. & Bibillo, A. (2009). Real-time DNA sequencing from single polymerase molecules. *Science*, *323*(5910), 133-138.

Ekwem, O. H. (2014). Isolation of antimicrobial producing lactobacilli from akamu (a Nigerian fermented cereal gruel). *African Journal of Microbiology Research*, *8*(7), 718-720.

El-Metwally, S., Hamza, T., Zakaria, M., & Helmy, M. (2013). Next-generation sequence assembly: four stages of data processing and computational challenges. *PLoS Computational Biology*, *9*(12), e1003345.

Enitan, A., Adeyemo, J., & Ogunbanwo, S. T. (2011). Influence of growth conditions and nutritional requirements on the production of hydrogen peroxide by lactic acid bacteria. *African Journal of Microbiology Research*, 5(15), 2059-2066.

Enujiugha, V. N., & Badejo, A. A. (2017). Probiotic potentials of cereal-based beverages. *Critical Reviews in Food Science and Nutrition*, *57*(4), 790-804.

Ercolini, D. (2013). High-throughput sequencing and metagenomics: moving forward in the culture-independent analysis of food microbial ecology. *Applied and Environmental Microbiology*, *79*(10), 3148-3155.

Ercolini, D., & Cocolin, L. S. (2014). Identification methods: Culture-independent techniques. *Elsevier*, 259-266.

Escalante, A., Wacher, C., & Farrés, A. (2001). Lactic acid bacterial diversity in the traditional Mexican fermented dough pozol as determined by 16S rDNA sequence analysis. *International Journal of Food Microbiology*, *64*(1-2), 21-31.

European Commission. (2010). Commission Regulation (EU) No 165/2010 of 26th February, 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. Official Journal of the European Union L50.

European Food Safety Authority. (2011). Evaluation of the FoodEx, the food classification system applied to the development of the EFSA Comprehensive European Food Consumption Database. *EFSA Journal*, *9*(3), 1970.

Ezekiel, C.N., Ayeni, K.I., Ezeokoli, O.T., Sulyok, M., Van Wyk, D.A., Oyedele, O.A., Akinyemi, O.M., Chibuzor-Onyema, I.E., Adeleke, R.A., Nwangburuka, C.C. & Hajšlová, J. (2019). High-throughput sequence analyses of bacterial communities and multi-mycotoxin profiling during processing of different formulations of Kunu, a traditional fermented beverage. *Frontiers in Microbiology*, *9*, 3282.

Fadahunsi, I. F., Garuba, E. O., Fawole, A. O., & Akinlawon, A. T. (2012). Production of Kenkey (A Ghanaian Starch-Based Food) Using Starter Cultures. *Journal of Food Technology*, *10*(4), 124-132.

Fasasi, O. S. (2009). Proximate, antinutritional factors and functional properties of processed pearl millet (Pennisetum glaucum). *Journal of Food Technology*, *7*(3), 92-97.

Fay, J. C., & Benavides, J. A. (2005). Evidence for domesticated and wild populations of Saccharomyces cerevisiae. *PLoS Genetics*, *1*(1), e5.

Feau, N., Decourcelle, T., Husson, C., Desprez-Loustau, M. L., & Dutech, C. (2011). Finding single copy genes out of sequenced genomes for multilocus phylogenetics in non-model fungi. *PLoS One*, *6*(4), e18803.

Felis, G. E., & Dellaglio, F. (2007). Taxonomy of lactobacilli and bifidobacteria. *Current Issues in Intestinal Microbiology*, *8*(2), 44-61.

Felix, A. S. H. (2016). A Review About Probiotic Foods: Kefir, Kimchi and Kombucha. *Journal of Food Process Technology*, *7*(11), 635-636.

Ferguson, R.M.W., Merrifield, D.L., Harper, G.M., Rawling, M.D., Mustafa, S., Picchietti, S., Balcàzar, J.L. & Davies, S.J. (2010). The effect of *Pediococcus acidilactici* on the gut microbiota and immune status of on-growing red tilapia (Oreochromis niloticus). *Journal of Applied Microbiology*, *109*(3), 851-862.

Ferri, M., Serrazanetti, D. I., Tassoni, A., Baldissarri, M., & Gianotti, A. (2016). Improving the functional and sensorial profile of cereal-based fermented foods by selecting Lactobacillus plantarum strains via a metabolomics approach. *Food Research International*, *89*, 1095-1105.

Fessard, A., & Remize, F. (2017). Why are Weissella spp. not used as commercial starter cultures for food fermentation?. *Fermentation*, *3*(3), 38.

Food and Agricultural Organization of the United Nations and World Health Organization. Joint FAO/WHO (2002). working group report on drafting guidelines for the evaluation of probiotics in food. Food and Agricultural Organization of the United Nations, ftp://ftp.fao.org/es/esn/food/wgreport2.pdf

Food and Agriculture Organization (2008). Sorghum and millets in Human Nutrition. FAO Food and Nutrition Series, No. 27. United Nations Food and Agriculture Organization, Rome.

Food and Agriculture Organization, ICRISAT. (1996). The World Sorghum and Millet Economies: Facts, Trends and Outlook. United Nations Food and Agriculture Organization, Rome, and International Crops Research Institute for the Semi-Arid Tropics, Andhra Pradesh, India.

Food and Agriculture Organization. (2016). FAO Report on Applications of Whole Genome Sequencing (WGS) in food safety management. www.fao.org/documents/card/ en/c/61e44b34-b328-4239-b59c-a9e926e327b4.

Foster-Nyarko, E., Alikhan, N.F., Ravi, A., Thilliez, G., Thomson, N.M., Baker, D., Kay, G., Cramer, J.D., O'Grady, J., Antonio, M. & Pallen, M.J. (2020). Genomic diversity of *Escherichia coli* isolates from non-human primates in the Gambia. *Microbial Genomics*, *6*(9).

França, L. T., Carrilho, E., & Kist, T. B. (2002). A review of DNA sequencing techniques. *Quarterly Reviews of Biophysics*, *35*(2), 169-200.

Francavilla, R., Polimeno, L., Demichina, A., Maurogiovanni, G., Principi, B., Scaccianoce, G., Ierardi, E., Russo, F., Riezzo, G., Di Leo, A. & Cavallo, L. (2014). *Lactobacillus reuteri* strain combination in Helicobacter pylori infection: a randomized, double-blind, placebo-controlled study. *Journal of Clinical Gastroenterology*, *48*(5), 407-413.

Frece, J., Kos, B., Svetec, I. K., Zgaga, Z., Beganović, J., Leboš, A., & Šušković, J. (2009). Synbiotic effect of Lactobacillus helveticus M92 and prebiotics on the intestinal microflora and immune system of mice. *Journal of Dairy Research*, *76*(1), 98-104.

Furukawa, S., Watanabe, T., Toyama, H., & Morinaga, Y. (2013). Significance of microbial symbiotic coexistence in traditional fermentation. *Journal of Bioscience and Bioengineering*, *116*(5), 533-539.

Fusco, V., Quero, G.M., Cho, G.S., Kabisch, J., Meske, D., Neve, H., Bockelmann, W. & Franz, C.M. (2015). The genus Weissella: taxonomy, ecology and biotechnological potential. *Frontiers in Microbiology*, *6*, 155.

Gabaza, M., Joossens, M., Cnockaert, M., Muchuweti, M., Raes, K., & Vandamme, P. (2019). Lactococci dominate the bacterial communities of fermented maize, sorghum and millet slurries in Zimbabwe. *International Journal of Food Microbiology*, *289*, 77-87.

Gadaga, T. H., Nyanga, L. K., & Mutukumira, A. N. (2004). The occurrence, growth and control of pathogens in African fermented foods. *African Jounal of Food, Agriculture, Nutrition and Development*, *4*, 5358-5374.

Gadaga, T. H., Samende, B. K., Musuna, C., & Chibanda, D. (2008). The microbiological quality of informally vended foods in Harare, Zimbabwe. *Food Control*, *19*(8), 829-832.

Galati, A., Oguntoyinbo, F. A., Moschetti, G., Crescimanno, M., & Settanni, L. (2014). The cereal market and the role of fermentation in cereal-based food production in Africa. *Food Reviews International*, *30*(4), 317-337.

Gänzle, M. G., & Vogel, R. F. (2003). Studies on the mode of action of reutericyclin. *Applied and Environmental Microbiology*, *69*(2), 1305-1307.

Gänzle, M. G., Loponen, J., & Gobbetti, M. (2008). Proteolysis in sourdough fermentations: mechanisms and potential for improved bread quality. *Trends in Food Science & Technology*, *19*(10), 513-521.

Gao, Y., Hou, L., Gao, J., Li, D., Tian, Z., Fan, B., Wang, F. & Li, S. (2021). Metabolomics Approaches for the Comprehensive Evaluation of Fermented Foods: A Review. *Foods*, *10*(10), 2294.

García-Hernández, Y., Rodríguez, Z., Brandão, L. R., Rosa, C. A., Nicoli, J. R., Iglesias, A. E., ... & Halaihel, N. (2012). Identification and in vitro screening of avian yeasts for use as probiotic. *Research in Veterinary Science*, *93*(2), 798-802.

Garsa, A. K., Choudhury, P. K., Puniya, A. K., Dhewa, T., Malik, R. K., & Tomar, S. K. (2019). Bovicins: the bacteriocins of streptococci and their potential in methane mitigation. *Probiotics and Antimicrobial Proteins*, *11*(4), 1403-1413.

Gatti, M., Trivisano, C., Fabrizi, E., Neviani, E., & Gardini, F. (2004). Biodiversity among Lactobacillus helveticus strains isolated from different natural whey starter cultures as revealed by classification trees. *Applied and Environmental Microbiology*, *70*(1), 182-190.

Gerez, C. L., Torino, M. I., Rollán, G., & de Valdez, G. F. (2009). Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food Control*, *20*(2), 144-148.

Ghana Standards Authority, GSA. (2013). Cereals, Pulses and derived products - Specification for pearl millet flour. (GS 728, Ghana Standards Authority, Accra, Ghana.

Ghrairi, T., Manai, M., Berjeaud, J. M., & Frere, J. (2004). Antilisterial activity of lactic acid bacteria isolated from rigouta, a traditional Tunisian cheese. *Journal of Applied Microbiology*, *97*(3), 621-628.

Gibson, G.R., Hutkins, R., Sanders, M.E., Prescott, S.L., Reimer, R.A., Salminen, S.J., Scott, K., Stanton, C., Swanson, K.S., Cani, P.D. & Verbeke, K. (2017). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature reviews Gastroenterology & Hepatology*, *14*(8), 491-502.

Gilbert, R. A. (2016). Strategies for improving ruminant utilisation of high grain diets: Pangenome of Streptococcus bovis. Department of Agriculture and Fisheries, Queensland.

Gille, D., Schmid, A., Walther, B., & Vergères, G. (2018). Fermented food and non-communicable chronic diseases: a review. *Nutrients*, *10*(4), 448.

Gil-Rodríguez, A. M., Carrascosa, A. V., & Requena, T. (2015). Yeasts in foods and beverages: In vitro characterisation of probiotic traits. *LWT-Food Science and Technology*, *64*(2), 1156-1162.

Gomes, A., & Korf, B. (2018). Genetic Testing Techniques. *Pediatric Cancer Genetics*, 47–64.

Gómez-Manzo, S., Chavez-Pacheco, J.L., Contreras-Zentella, M., Sosa-Torres, M.E., Arreguín-Espinosa, R., Perez De La Mora, M., Membrillo-Hernández, J. & Escamilla, J.E. (2010). Molecular and catalytic properties of the aldehyde dehydrogenase of *Gluconacetobacter diazotrophicus*, a quinoheme protein containing pyrroloquinoline quinone, cytochrome b, and cytochrome c. *Journal of Bacteriology*, *192*(21), 5718-5724.

Gomis, B. D. (1992). HPLC analysis of organic acids. *Food Analysis by HPLC. Marcel Dekker Inc. New York, USA*, *37*(1), 358.

Gonçalves, B. L., Rosim, R. E., de Oliveira, C. A. F., & Corassin, C. H. (2015). The in vitro ability of different Saccharomyces cerevisiae–based products to bind aflatoxin B1. *Food Control*, *47*, 298-300.

Górska, S., Grycko, P., Rybka, J., & Gamian, A. (2007). Egzopolisacharydy bakterii kwasu mlekowego–biosynteza i struktura Exopolysaccharides of lactic acid bacteria: Structure and biosynthesis. *Postepy Hig. Med. Dosw.(Online)*, *61*, 805-818.

Gotcheva, V., Hristozova, E., Hristozova, T., Guo, M., Roshkova, Z., & Angelov, A. (2002). Assessment of potential probiotic properties of lactic acid bacteria and yeast strains. *Food Biotechnology*, *16*(3), 211-225.

Gourama, H., & Bullerman, L. B. (1995). Inhibition of growth and aflatoxin production of Aspergillus flavus by Lactobacillus species. *Journal of Food Protection*, *58*(11), 1249-1256.

Gravesen, A., Diao, Z., Voss, J., Budde, B. B., & Knøchel, S. (2004). Differential inactivation of Listeria monocytogenes by d-and l-lactic acid. *Letters in Applied Microbiology*, *39*(6), 528-532.

Greppi, A., Rantsiou, K., Padonou, W., Hounhouigan, J., Jespersen, L., Jakobsen, M., & Cocolin, L. (2013). Determination of yeast diversity in ogi, mawè, gowé and tchoukoutou by using culture-dependent and-independent methods. *International Journal of Food Microbiology*, *165*(2), 84-88.

Greppi, A., Saubade, F., Botta, C., Humblot, C., Guyot, J. P., & Cocolin, L. (2017). Potential probiotic Pichia kudriavzevii strains and their ability to enhance folate content of traditional cereal-based African fermented food. *Food Microbiology*, *62*, 169-177.

Gueimonde, M., Jalonen, L., He, F., Hiramatsu, M., & Salminen, S. (2006). Adhesion and competitive inhibition and displacement of human enteropathogens by selected lactobacilli. *Food Research International*, *39*(4), 467-471.

Gugliandolo, C., Lentini, V., Spanò, A., & Maugeri, T. L. (2011). Conventional and molecular methods to detect bacterial pathogens in mussels. *Letters in Applied Microbiology*, *52*(1), 15-21.

Guiraud, J. P. (1998). *Microbiologie Alimentaire*. Dunod. Paris. France.

Gullo, M., Verzelloni, E., & Canonico, M. (2014). Aerobic submerged fermentation by acetic acid bacteria for vinegar production: process and biotechnological aspects. *Process Biochemistry*, *49*(10), 1571-1579.

Gutierrez-Castrellon, P., Lopez-Velazquez, G., Diaz-Garcia, L., Jimenez-Gutierrez, C., Mancilla-Ramirez, J., Estevez-Jimenez, J., & Parra, M. (2014). Diarrhea in preschool children and Lactobacillus reuteri: a randomized controlled trial. *Pediatrics*, *133*(4), e904-e909.

Guzvic, M. (2013). The History of DNA Sequencing/ISTORIJAT SEKVENCIRANJA DNK. *Journal of Medical Biochemistry*, *32*(4), 301-312.

Hagemann, I. S. (2015). Overview of technical aspects and chemistries of next-generation sequencing. *Clinical Genomics*, 3-19.

Haleegoah, J. A. S., Ruivenkamp, G., Essegbey, G., Frempong, G., & Jongerden, J. (2016). Street-vended local foods transformation: case of hausa koko, waakye and ga kenkey in urban Ghana. *Advances in Applied Sociology*, *6*(3), 90-100.

Haleegoah, J., Ruivenkamp, G., Essegbey, G., Frempong, G., & Jongerden, J. (2015). Street-Vended Local Food Systems Actors Perceptions on Safety in Urban Ghana: The Case of Hausa Koko, Waakye and Ga Kenkey. *Advances in Applied Sociology*, *5*(04), 134.

Hall, N. (2007). Advanced sequencing technologies and their wider impact in microbiology. *Journal of Experimental Biology*, *210*(9), 1518-1525.

Halm, M., Amoa-Awua, W. K., & Jakobsen, M. (2004). Kenkey: an African fermented maize product. *Handbook of Food and Beverage Fermentation Technology*, 799-816.

Halm, M., Lillie, A., Sørensen, A. K., & Jakobsen, M. (1993). Microbiological and aromatic characteristics of fermented maize doughs for kenkey production in Ghana. *International Journal of Food Microbiology*, *19*(2), 135-143.

Halm, M., Osei-Yaw, A., Hayford, A., Kpodo, K. A., & Amoa-Awua, W. K. A. (1996). Experiences with the use of a starter culture in the fermentation of maize for 'kenkey'production in Ghana. *World Journal of Microbiology and Biotechnology*, *12*(5), 531-536.

Hammes, W. P., & Hertel, C. (2006). The genera lactobacillus and carnobacterium. *Prokaryotes*, *4*, 320-403.

Hardalo, C., & Edberg, S. C. (1997). Pseudomonas aeruginosa: assessment of risk from drinking water. *Critical Reviews in Microbiology*, *23*(1), 47-75.

Head, I. M., Saunders, J. R., & Pickup, R. W. (1998). Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbial Ecology*, *35*(1), 1-21.

Henshaw, E., Akpan, J., Lennox, J., & Frank, E. (2016). Bacterial community dynamics during the production of ogi from millet 'a Nigerian fermented food'using culture-dependent approach. *Imperial. Journal of Interdisciplinary Research*, *2*, 621-623.

Hernández-González, J. C., Martínez-Tapia, A., Lazcano-Hernández, G., García-Pérez, B. E., & Castrejón-Jiménez, N. S. (2021). Bacteriocins from Lactic Acid Bacteria. A Powerful Alternative as Antimicrobials, Probiotics, and Immunomodulators in Veterinary Medicine. *Animals*, *11*(4), 979.

Herreros, M. A., Sandoval, H., González, L., Castro, J. M., Fresno, J. M., & Tornadijo, M. E. (2005). Antimicrobial activity and antibiotic resistance of lactic acid bacteria isolated from Armada cheese (a Spanish goats' milk cheese). *Food Microbiology*, *22*(5), 455-459.

Hertzberger, R., Arents, J., Dekker, H. L., Pridmore, R. D., Gysler, C., Kleerebezem, M., & de Mattos, M. J. T. (2014). H2O2 production in species of the *Lactobacillus acidophilus* group: a central role for a novel NADH-dependent flavin reductase. *Applied and Environmental Microbiology*, *80*(7), 2229-2239.

Hilton, A. C., & Austin, E. (2000). The kitchen dishcloth as a source of and vehicle for foodborne pathogens in a domestic setting. *International Journal of Environmental Health Research*, *10*(3), 257-261.

Hinton Jr, A. (2006). Growth of Campylobacter in media supplemented with organic acids. *Journal of Food Protection*, *69*(1), 34-38.

Hoff, K. J., Tech, M., Lingner, T., Daniel, R., Morgenstern, B., & Meinicke, P. (2008). Gene prediction in metagenomic fragments: a large scale machine learning approach. *BMC Bioinformatics*, *9*(1), 1-14.

Hofvendahl, K., & Hahn–Hägerdal, B. (2000). Factors affecting the fermentative lactic acid production from renewable resources1. *Enzyme and Microbial Technology*, *26*(2-4), 87-107.

Holt, S., Mukherjee, V., Lievens, B., Verstrepen, K. J., & Thevelein, J. M. (2018). Bioflavoring by non-conventional yeasts in sequential beer fermentations. *Food Microbiology*, *72*, 55-66.

Holzapfel, W. (1997). Use of starter cultures in fermentation on a household scale. *Food Control*, *8*(5-6), 241-258.

Holzapfel, W. H. (2002). Appropriate starter culture technologies for small-scale fermentation in developing countries. *International Journal of Food Microbiology*, *75*(3), 197-212.

Horner, D.S., Pavesi, G., Castrignano, T., De Meo, P.D.O., Liuni, S., Sammeth, M., Picardi, E. & Pesole, G. (2010). Bioinformatics approaches for genomics and post genomics applications of next-generation sequencing. *Briefings in Bioinformatics*, *11*(2), 181-197.

Houngbédji, M., Johansen, P., Padonou, S.W., Akissoé, N., Arneborg, N., Nielsen, D.S., Hounhouigan, D.J. & Jespersen, L. (2018). Occurrence of lactic acid bacteria and yeasts at species and strain level during spontaneous fermentation of mawè, a cereal dough produced in West Africa. *Food Microbiology*, *76*, 267-278.

Hu, C., & Xu, G. (2013). Mass-spectrometry-based metabolomics analysis for foodomics. *TrAC Trends in Analytical Chemistry*, *52*, 36-46.

Humblot, C., & Guyot, J. P. (2009). Pyrosequencing of tagged 16S rRNA gene amplicons for rapid deciphering of the microbiomes of fermented foods such as pearl millet slurries. *Applied and Environmental Microbiology*, *75*(13), 4354-4361.

Indrio, F., Di Mauro, A., Riezzo, G., Civardi, E., Intini, C., Corvaglia, L., Ballardini, E., Bisceglia, M., Cinquetti, M., Brazzoduro, E. & Del Vecchio, A. (2014). Prophylactic use of a probiotic in the prevention of colic, regurgitation, and functional constipation: a randomized clinical trial. *JAMA Pediatrics*, *168*(3), 228-233.

International Organization for Standards (ISO). (2008). Horizontal method for the enumeration of yeasts and moulds. Method No 21527-1:2008

Ito, A., Sato, Y., Kudo, S., Sato, S., Nakajima, H., & Toba, T. (2003). The screening of hydrogen peroxide-producing lactic acid bacteria and their application to inactivating psychrotrophic food-borne pathogens. *Current Microbiology*, *47*(3), 0231-0236.

Iyer, B. K., & Ananthanarayan, L. (2008). Effect of α-amylase addition on fermentation of idli—a popular south Indian cereal—Legume-based snack food. *LWT-Food Science and Technology*, *41*(6), 1053-1059.

Izah, S. C., Kigigha, L. T., & Okowa, I. P. (2016). Microbial quality assessment of fermented maize Ogi (a cereal product) and options for overcoming constraints in production. *Biotechnological Research*, *2*(2), 81-93.

Jacobsen, C.N., Rosenfeldt Nielsen, V., Hayford, A.E., Møller, P.L., Michaelsen, K.F., Paerregaard, A., Sandstrom, B., Tvede, M. & Jakobsen, M. (1999). Screening of probiotic activities of forty-seven strains of Lactobacillus spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. *Applied and Environmental Microbiology*, *65*(11), 4949-4956.

Jalaja, N., Maheshwari, P., Naidu, K. R., & Kavi Kishor, P. B. (2016). In vitro regeneration and optimization of conditions for transformation methods in Pearl millet, Pennisetum glaucum (L.). *International Journal of Clinical and Biological Sciences*, *1*(1), 34-52.

Jan, G., Leverrier, P., Pichereau, V., & Boyaval, P. (2001). Changes in protein synthesis and morphology during acid adaptation of Propionibacterium freudenreichii. *Applied and Environmental Microbiology*, *67*(5), 2029-2036.

Janssens, L., De Pooter, H. L., Schamp, N. M., & Vandamme, E. J. (1992). Production of flavours by microorganisms. *Process Biochemistry*, *27*(4), 195-215.

Jaybhaye, R. V., Pardeshi, I. L., Vengaiah, P. C., & Srivastav, P. P. (2014). Processing and technology for millet based food products: a review. *Journal of Ready to Eat Food*, *1*(2), 32-48.

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

Jespersen, L. (2003). Occurrence and taxonomic characteristics of strains of Saccharomyces cerevisiae predominant in African indigenous fermented foods and beverages. *FEMS Yeast Research*, *3*(2), 191-200.

Jespersen, L., Halm, M., Kpodo, K., & Jakobsen, M. (1994). Significance of yeasts and moulds occurring in maize dough fermentation for 'kenkey' production. *International Journal of Food Microbiology*, *24*(1-2), 239-248.

Jespersen, L., Nielsen, D. S., Hønholt, S., & Jakobsen, M. (2005). Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans. *FEMS Yeast Research*, *5*(4-5), 441-453.

Jiang, S., Cai, L., Lv, L., & Li, L. (2021). Pediococcus pentosaceus, a future additive or probiotic candidate. *Microbial Cell Factories*, *20*(1), 1-14.

Johansen, P. G., Owusu-Kwarteng, J., Parkouda, C., Padonou, S. W., & Jespersen, L. (2019). Occurrence and importance of yeasts in indigenous fermented food and beverages produced in Sub-Saharan Africa. *Frontiers in Microbiology*, *10*, 1789.

Johnson, E. A. (2013). Biotechnology of non-Saccharomyces yeasts—the ascomycetes. *Applied Microbiology and Biotechnology*, *97*(2), 503-517.

Jung, J.Y., Lee, S.H., Kim, J.M., Park, M.S., Bae, J.W., Hahn, Y., Madsen, E.L. & Jeon, C.O. (2011). Metagenomic analysis of kimchi, a traditional Korean fermented food. *Applied and Environmental Microbiology*, *77*(7), 2264-2274.

Kalui, C., Mathara, J., Kutima, P., Kiiyukia, C., & Wongo, L. (2009). Functional characteristics of Lactobacillus plantarum and Lactobacillus rhamnosus from ikii, a Kenyan traditional fermented maize porridge. *African Journal of Biotechnology*, *8*(18), 4363–4373.

Kandler, O. & Weiss, N. (1986). Genus Lactobacillus Beijerinck 1901, 212AL. In Bergey's Manual of Systematic Bacteriology, vol. 2, pp. 1209–1234. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.

Kang, H. J., Yang, H. J., Kim, M. J., Han, E. S., Kim, H. J., & Kwon, D. Y. (2011). Metabolomic analysis of meju during fermentation by ultra performance liquid chromatography-quadrupole-time of flight mass spectrometry (UPLC-Q-TOF MS). *Food Chemistry*, *127*(3), 1056-1064.

Kanton, R.A.L., Asungre, P., Ansoba, E.Y., Inusah, B.I., Bidzakin, J.K., Abubakari, M. & Akum, F.A. (2015). Evaluation of pearl millet varieties for adaptation to the semi-arid agro-ecology of northern Ghana. *Journal of Agriculture and Ecology Research International*, 1-11.

Karovičová, Z. K. J., & Kohajdova, J. (2007). Fermentation of cereals for specific purpose. *Journal of Food and Nutrition Research*, *46*(2), 51-57.

Kellis, M., Patterson, N., Endrizzi, M., Birren, B., & Lander, E. S. (2003). Sequencing and comparison of yeast species to identify genes and regulatory elements. *Nature*, *423*(6937), 241-254.

Kigigha, L. T., Izah, S. C., & Okowa, I. P. (2016). Microbial Characterisation of Maize Fermentation Water during Ogi Production. *Journal of Environmental Treatment Techniques*, *4*, 41-45.

Kim, H. K., Choi, Y. H., & Verpoorte, R. (2010). NMR-based metabolomic analysis of plants. *Nature Protocols*, *5*(3), 536-549.

Kimaryo, V. M., Massawe, G. A., Olasupo, N. A., & Holzapfel, W. H. (2000). The use of a starter culture in the fermentation of cassava for the production of "kivunde", a traditional Tanzanian food product. *International Journal of Food Microbiology*, *56*(2-3), 179-190.

Knoshaug, E. P., Ahlgren, J. A., & Trempy, J. E. (2000). Growth associated exopolysaccharide expression in Lactococcus lactis subspecies cremoris Ropy352. *Journal of Dairy Science*, *83*(4), 633-640.

Koboldt, D. C., Steinberg, K. M., Larson, D. E., Wilson, R. K., & Mardis, E. R. (2013). The next-generation sequencing revolution and its impact on genomics. *Cell*, *155*(1), 27-38.

Kot, E., Furmanov, S., & Bezkorovainy, A. (1996). Hydrogen peroxide production and oxidation of ferrous iron by Lactobacillus delbrueckii ssp. bulgaricus. *Journal of Dairy Science*, *79*(5), 758-766.

Kovárová-Kovar, K., & Egli, T. (1998). Growth kinetics of suspended microbial cells: from single-substrate-controlled growth to mixed-substrate kinetics. *Microbiology and Molecular Biology Reviews*, *62*(3), 646-666.

Kowallik, V., Miller, E., & Greig, D. (2015). The interaction of Saccharomyces paradoxus with its natural competitors on oak bark. *Molecular Ecology*, *24*(7), 1596-1610.

Koziolek, M., Grimm, M., Becker, D., Iordanov, V., Zou, H., Shimizu, J., ... & Weitschies, W. (2015). Investigation of pH and temperature profiles in the GI tract of fasted human subjects using the Intellicap® system. *Journal of pharmaceutical sciences*, *104*(9), 2855-2863.

Kpodo, K., Sørensen, A. K., & Jakobsen, M. (1996). The occurrence of mycotoxins in fermented maize products. *Food Chemistry*, *56*(2), 147-153.

Kulski, J. K. (2016). Next-generation sequencing—an overview of the history, tools, and "Omic" applications. *Next generation sequencing–advances, applications and challenges*, *3*, 60.

Kumar, A. S., & Mody, K. (2009). Microbial exopolysaccharides: variety and potential applications. *Microbial Production of Biopolymers and Polymer Precursors: Applications and Perspectives*, 229-253.

Kumar, B. V., Umamahesh, K., & Reddy, O. V. S. (2017). Isolation and Identification of Pediococcus pentosaceus from Cow's Milk Curd and Its Use in Grape Juices Fermentation. *Journal of Advances in Biology & Biotechnology*, 1-11.

Kumar, V., Sinha, A. K., Makkar, H. P., & Becker, K. (2010). Dietary roles of phytate and phytase in human nutrition: A review. *Food Chemistry*, *120*(4), 945-959.

Kusumaningrum, H. D., Riboldi, G., Hazeleger, W. C., & Beumer, R. R. (2003). Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. *International Journal of Food Microbiology*, *85*(3), 227-236.

Laca, A., Mousia, Z., Díaz, M., Webb, C., & Pandiella, S. S. (2006). Distribution of microbial contamination within cereal grains. *Journal of Food Engineering*, *72*(4), 332-338.

Land, M., Hauser, L., Jun, S. R., Nookaew, I., Leuze, M. R., Ahn, T. H., ... & Ussery, D. W. (2015). Insights from 20 years of bacterial genome sequencing. *Functional & Integrative Genomics*, *15*(2), 141-161.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L., & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences*, *82*(20), 6955-6959.

Lee, D. S. (2016). Carbon dioxide absorbers for food packaging applications. *Trends in Food Science & Technology*, *57*, 146-155.

Lee, K. W., Park, J. Y., Jeong, H. R., Heo, H. J., Han, N. S., & Kim, J. H. (2012). Probiotic properties of Weissella strains isolated from human faeces. *Anaerobe*, *18*(1), 96-102.

Lei, V., & Jakobsen, M. (2004). Microbiological characterisation and probiotic potential of koko and koko sour water, African spontaneously fermented millet porridge and drink. *Journal of Applied Microbiology*, *96*(2), 384-397.

Lei, V., Friis, H., & Michaelsen, K. F. (2006). Spontaneously fermented millet product as a natural probiotic treatment for diarrhoea in young children: an intervention study in Northern Ghana. *International Journal of Food Microbiology*, *110*(3), 246-253.

Lestienne, I., Buisson, M., Lullien-Pellerin, V., Picq, C., & Trèche, S. (2007). Losses of nutrients and anti-nutritional factors during abrasive decortication of two pearl millet cultivars (Pennisetum glaucum). *Food Chemistry*, *100*(4), 1316-1323.

Li, M., Wang, H., Zhao, G., Qiao, M., Li, M., Sun, L., Gao, X. & Zhang, J. (2014). Determining the drying degree and quality of chicken jerky by LF-NMR. *Journal of Food Engineering*, *139*, 43-49.

Lin, H., Bennett, G. N., & San, K. Y. (2005). Metabolic engineering of aerobic succinate production systems in Escherichia coli to improve process productivity and achieve the maximum theoretical succinate yield. *Metabolic Engineering*, *7*(2), 116-127.

Lindgren, S. E., & Dobrogosz, W. J. (1990). Antagonistic activities of lactic acid bacteria in food and feed fermentations. *FEMS Microbiology Reviews*, *7*(1-2), 149-163.

Liu, Z., DeSantis, T. Z., Andersen, G. L., & Knight, R. (2008). Accurate taxonomy assignments from 16S rRNA sequences produced by highly parallel pyrosequencers. *Nucleic Acids Research*, *36*(18), e120-e120.

Loman, N. J., & Pallen, M. J. (2015). Twenty years of bacterial genome sequencing. *Nature Reviews Microbiology*, *13*(12), 787-794.

López-Cuellar, M. D. R., Rodríguez-Hernández, A. I., & Chavarría-Hernández, N. (2016). LAB bacteriocin applications in the last decade. *Biotechnology & Biotechnological Equipment*, *30*(6), 1039-1050.

Lu, J., Shi, Y., Wang, S., Chen, H., Cai, S., & Feng, J. (2016). NMR-based metabolomic analysis of Haliotis diversicolor exposed to thermal and hypoxic stresses. *Science of the Total Environment*, *545*, 280-288.

Macfarlane S., Macfarlane G.T. & Cummings J.H., (2006). Review article: prebiotics in the gastrointestinal tract. *Alimentary Pharmacology & Therapeutics*, *24*(5), 701-714.

Madigan, M. T., Martinko, J. M., Stahl, D. A., & Clark, D. P. (2012). Cell structure and function in Bacteria and Archaea. *Brock Biology of Microorganisms*, *49*.

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., ... & Mills, D. (2006). Comparative genomics of the lactic acid bacteria. *Proceedings of the National Academy of Sciences*, *103*(42), 15611-15616.

Malleshi, N. G., & Klopfenstein, C. F. (1998). Nutrient composition and amino acid contents of malted sorghum, pearl millet and finger millet and their milling fractions. *Journal of Food Science and Technology*, *35*(3), 247-249.

Mani, A. (2018). Food Preservation by Fermentation and Fermented food products. *International Journal of Academic Research & Development*, *1*, 51-57.

Manini, F., Casiraghi, M. C., Poutanen, K., Brasca, M., Erba, D., & Plumed-Ferrer, C. (2016). Characterisation of lactic acid bacteria isolated from wheat bran sourdough. *LWT-Food Science and Technology*, *66*, 275-283.

Marchesi, J. R., Adams, D. H., Fava, F., Hermes, G. D., Hirschfield, G. M., Hold, G., ... & Hart, A. (2016). The gut microbiota and host health: a new clinical frontier. *Gut*, *65*(2), 330-339.

Marieb, E. N., & Hoehn, K. (2018). *Human anatomy & physiology*. Pearson education, 11th Ed. Inc. p. 1264. ISBN 0134580990.

Marin, S., Ramos, A. J., Cano-Sancho, G., & Sanchis, V. (2013). Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology*, *60*, 218-237.

Marsh, A. J., Hill, C., Ross, R. P., & Cotter, P. D. (2014). Fermented beverages with health-promoting potential: Past and future perspectives. *Trends in Food Science & Technology*, *38*(2), 113-124.

Maukonen, J., Mättö, J., Wirtanen, G., Raaska, L., Mattila-Sandholm, T., & Saarela, M. (2003). Methodologies for the characterisation of microbes in industrial environments: a review. *Journal of Industrial Microbiology and Biotechnology*, *30*(6), 327-356.

Mayra-Makinen, A., & Bigret, M. (2004). Industrial use and production of lactic acid bacteria. *Food Science and Technology-New York-Marcel Dekker-*, *139*, 175-198.

Mbithi-Mwikya, S., Ooghe, W., Van Camp, J., Ngundi, D., & Huyghebaert, A. (2000). Amino acid profiles after sprouting, autoclaving, and lactic acid fermentation of finger millet (Eleusine coracan) and kidney beans (Phaseolus vulgaris L.). *Journal of Agricultural and Food Chemistry*, *48*(8), 3081-3085.

McAllister, T. A., Beauchemin, K. A., Alazzeh, A. Y., Baah, J., Teather, R. M., & Stanford, K. (2011). The use of direct fed microbials to mitigate pathogens and enhance production in cattle. *Canadian Journal of Animal Science*, *91*(2), 193-211.

McEntire, J. C., Montville, T. J., & Chikindas, M. L. (2003). Synergy between nisin and select lactates against Listeria monocytogenes is due to the metal cations. *Journal of Food Protection*, *66*(9), 1631-1636.

McFarland, L. V. (2007). Meta-analysis of probiotics for the prevention of traveler's diarrhea. *Travel Medicine and Infectious Disease*, *5*(2), 97-105.

Mechai, A., Debabza, M., & Kirane, D. (2014). Screening of technological and probiotic properties of lactic acid bacteria isolated from Algerian traditional fermented milk products. *International Food Research Journal*, *21*(6).

Mensah, P. (1997). Fermentation—the key to food safety assurance in Africa?. *Food Control*, *8*(5-6), 271-278.

Mensah, P. P., Tomkins, A. M., Drasar, B. S., & Harrison, T. J. (1990). Fermentation of cereals for reduction of bacterial contamination of weaning foods in Ghana. *The Lancet*, *336*(8708), 140-143.

Mensah, P., Tomkins, A. M., Drasar, B. S., & Harrison, T. J. (1991). Antimicrobial effect of fermented Ghanaian maize dough. *Journal of Applied Bacteriology*, *70*(3), 203-210.

Messens, W., & De Vuyst, L. (2002). Inhibitory substances produced by Lactobacilli isolated from sourdoughs—a review. *International Journal of Food Microbiology*, *72*(1-2), 31-43.

Metzker, M. L. (2005). Emerging technologies in DNA sequencing. *Genome Research*, *15*(12), 1767-1776.

Metzker, M. L. (2010). Sequencing technologies—the next generation. *Nature Reviews Genetics*, *11*(1), 31-46.

Michodjèhoun-Mestres, L., Hounhouigan, D. J., Dossou, J., & Mestres, C. (2005). Physical, chemical and microbiological changes during natural fermentation of\" gowé\", a sprouted or non sprouted sorghum beverage from West-Africa. *African Journal of Biotechnology*, *4*(6), 487-496.

Min, J. S., Lee, S. O., Jang, A., Jo, C., & Lee, M. (2007). Irradiation and organic acid treatment for microbial control and the production of biogenic amines in beef and pork. *Food Chemistry*, *104*(2), 791-799.

Mishra, S. S., Ray, R. C., Panda, S. K., & Montet, D. (2017). Technological innovations in processing of fermented foods an overview. *Fermented Foods*, 21-45.

Misihairabgwi, J. M., Ishola, A., Quaye, I., Sulyok, M., & Krska, R. (2018). Diversity and fate of fungal metabolites during the preparation of oshikundu, a Namibian traditional fermented beverage. *World Mycotoxin Journal*, *11*(3), 471-481.

Mohamed, E. A., Ali, N. A., Ahmed, S. H., Ahmed, I. A. M., & Babiker, E. E. (2010). Effect of radiation process on antinutrients and HCl extractability of calcium, phosphorus and iron during processing and storage. *Radiation Physics and Chemistry*, *79*(7), 791-796.

Mohania, D., Nagpal, R., Kumar, M., Bhardwaj, A., Yadav, M., Jain, S., ... & Yadav, H. (2008). Molecular approaches for identification and characterisation of lactic acid bacteria. *Journal of Digestive Diseases*, *9*(4), 190-198.

Mokoena, M. P., Mutanda, T., & Olaniran, A. O. (2016). Perspectives on the probiotic potential of lactic acid bacteria from African traditional fermented foods and beverages. *Food & Nutrition Research*, *60*(1), 29630.

Mollakhalili Meybodi, N., & Mohammadifar, M. A. (2015). Microbial exopolysaccharides: a review of their function and application in food sciences. *Journal of Food Quality and Hazards Control*, *2*(4), 112-117.

Monnet, C., El Attar, A., & Corrieu, G. (2002). Production of carbon dioxide by Lactococcus lactis strains with attenuated lactate dehydrogenase activity, in pure cultures and in mixed cultures with an acidifying strain. *Le Lait*, *82*(3), 267-279.

Moratalla-López, N., Bagur, M. J., Lorenzo, C., Martínez-Navarro, M. E., Salinas, M. R., & Alonso, G. L. (2019). Bioactivity and bioavailability of the major metabolites of Crocus sativus L. Flower. *Molecules*, *24*(15), 2827.

Morelli, L., & Capurso, L. (2012). FAO/WHO guidelines on probiotics: 10 years later. *Journal of Clinical Gastroenterology*, *46*, S1-S2.

Morozova, O., & Marra, M. A. (2008). Applications of next-generation sequencing technologies in functional genomics. *Genomics*, *92*(5), 255-264.

Motarjemi, Y., & Nout, M. J. (1996). Food fermentation: a safety and nutritional assessment/Y. Motarjemi & MJR Nout on behalf of the Joint FAO/WHO Workshop on Assessment of Fermentation as a Household Technology for Improving Food Safety. Bulletin of the World Health Organization, 74 (6): 553-559.

Mozzi, F., Ortiz, M. E., Bleckwedel, J., De Vuyst, L., & Pescuma, M. (2013). Metabolomics as a tool for the comprehensive understanding of fermented and functional foods with lactic acid bacteria. *Food Research International*, *54*(1), 1152-1161.

Mu, Q., Tavella, V. J., & Luo, X. M. (2018). Role of *Lactobacillus reuteri* in human health and diseases. *Frontiers in Microbiology*, *9*, 757.

Mugula, J. K., Nnko, S. A. M., Narvhus, J. A., & Sørhaug, T. (2003a). Microbiological and fermentation characteristics of togwa, a Tanzanian fermented food. *International Journal of Food Microbiology*, *80*(3), 187-199.

Mugula, J. K., Narvhus, J. A., & Sørhaug, T. (2003b). Use of starter cultures of lactic acid bacteria and yeasts in the preparation of togwa, a Tanzanian fermented food. *International Journal of Food Microbiology*, *83*(3), 307-318.

Mukisa, I. M., Porcellato, D., Byaruhanga, Y. B., Muyanja, C. M., Rudi, K., Langsrud, T., & Narvhus, J. A. (2012). The dominant microbial community associated with fermentation of Obushera (sorghum and millet beverages) determined by culture-dependent and culture-independent methods. *International Journal of Food Microbiology*, *160*(1), 1-10.

Muñoz, R., Arena, M. E., Silva, J., & Gonzalez, S. N. (2010). Inhibition of mycotoxin-producing Aspergillus nomius VSC 23 by lactic acid bacteria and Saccharomyces cerevisiae. *Brazilian Journal of Microbiology*, *41*(4), 1019-1026.

Munroe, D. J., & Harris, T. J. (2010). Third-generation sequencing fireworks at Marco Island. *Nature Biotechnology*, *28*(5), 426-428.

Mustapha, M. B., Bousselmi, M., Jerbi, T., Bettaïeb, N. B., & Fattouch, S. (2014). Gamma radiation effects on microbiological, physico-chemical and antioxidant properties of Tunisian millet (Pennisetum Glaucum LR Br.). *Food Chemistry*, *154*, 230-237.

Muyanja, C. M. B. K., Narvhus, J. A., Treimo, J., & Langsrud, T. (2003). Isolation, characterisation and identification of lactic acid bacteria from bushera: a Ugandan traditional fermented beverage. *International Journal of Food Microbiology*, *80*(3), 201-210.

Nagpal, R., & Kaur, A. (2011). Synbiotic effect of various prebiotics on in vitro activities of probiotic lactobacilli. *Ecology of Food and Nutrition*, *50*(1), 63-68.

Nagpal, R., Kumar, A., Kumar, M., Behare, P. V., Jain, S., & Yadav, H. (2012). Probiotics, their health benefits and applications for developing healthier foods: a review. *FEMS Microbiology Letters*, *334*(1), 1-15.

Nagpal, R., Yadav, H., Puniya, A. K., Singh, K., Jain, S., & Marotta, F. (2007). Potential of probiotics and prebiotics for synbiotic functional dairy foods: an overview. *International Journal of Probiotics and Prebiotics*, *2*(2/3), 75-84.

Nakatsu, C. H., Byappanahalli, M. N., & Nevers, M. B. (2019). Bacterial community 16S rRNA gene sequencing characteries riverine microbial impact on Lake Michigan. *Frontiers in Microbiology*, *10*, 996.

Nam, H., Ha, M., Bae, O., & Lee, Y. (2002). Effect of Weissella confusa strain PL9001 on the adherence and growth of Helicobacter pylori. *Applied and Environmental Microbiology*, *68*(9), 4642-4645.

Nami, Y., Gharekhani, M., Aalami, M., & Hejazi, M. A. (2019). Lactobacillus-fermented sourdoughs improve the quality of gluten-free bread made from pearl millet flour. *Journal of Food Science and Technology*, *56*(9), 4057-4067.

National Institutes of Health. (2015). 2,3-Butanedione. U.S. National Libraryof Medicine, National Center for Biotechnology Information, PubChemOpen Chemistry Database.

National Research Council, (NRC). (1992). Applications of biotechnology in traditional fermented foods. National Academies Press.

Naumova, E. S., Korshunova, I. V., Jespersen, L., & Naumov, G. I. (2003). Molecular genetic identification of Saccharomyces sensu stricto strains from African sorghum beer. *FEMS Yeast Research*, *3*(2), 177-184.

Nayak, S. K. (2011). Biology of eukaryotic probiotics. In *Probiotics* (pp. 29-55). Springer, Berlin, Heidelberg.

Nazir, A. (2016). Review on metagenomics and its applications. *Imperial Journal of Interdisciplinary Research*, *2*(10).

Neefs, J. M., Van de Peer, Y., De Rijk, P., Chapelle, S., & De Wachter, R. (1993). Compilation of small ribosomal subunit RNA structures. *Nucleic Acids Research*, *21*(13), 3025-3049.

Nes, I. F., & Johnsborg, O. (2004). Exploration of antimicrobial potential in LAB by genomics. *Current Opinion in Biotechnology*, *15*(2), 100-104.

Ng, P. C., & Kirkness, E. F. (2010). Whole genome sequencing. *Genetic variation*, 215-226.

Nieminen, M.T., Novak-Frazer, L., Collins, R., Dawsey, S.P., Dawsey, S.M., Abnet, C.C., White, R.E., Freedman, N.D., Mwachiro, M., Bowyer, P. & Salaspuro, M. (2013). Alcohol and acetaldehyde in African fermented milk mursik—a possible etiologic factor for high incidence of esophageal cancer in western Kenya. *Cancer Epidemiology and Prevention Biomarkers*, *22*(1), 69-75.

Nilsen, T., Nes, I. F., & Holo, H. (2003). Enterolysin A, a cell wall-degrading bacteriocin from *Enterococcus faecalis* LMG 2333. *Applied and Environmental Microbiology*, *69*(5), 2975-2984.

Njeru, P., Rösch, N., Ghadimi, D., Geis, A., Bockelmann, W., de Vrese, M., Schrezenmeir, J. & Heller, K. (2010). Identification and characterisation of lactobacilli isolated from Kimere, a spontaneously fermented pearl millet dough from Mbeere, Kenya (East Africa). *Beneficial Microbes*, *1*(3), 243-252.

Nkhata, S. G., Ayua, E., Kamau, E. H., & Shingiro, J. B. (2018). Fermentation and germination improve nutritional value of cereals and legumes through activation of endogenous enzymes. *Food Science & Nutrition*, *6*(8), 2446-2458.

Nnam, N. M. (2001). Chemical, sensory and rheological properties of porridges from processed sorghum (Sorghum bicolor, bambara groundnut (Vigna subterranea L. Verdc) and sweet potato (Ipomoea batatas) flours. *Plant Foods for Human Nutrition*, *56*(3), 251-264.

NMKL (1999). *Salmonella* Detection in Foods. Method No. 71, 5th ed. Nordic Committee on Food Analysis, Oslo, Norway.

NMKL (1999). Aerobic Plate Count in Foods. Method No. 86, Nordic Committee on Food Analysis Method. (1999). Oslo, Norway.

NMKL (2003). *Staphylococcus aureus* Counts in Foods. Method No. 66, 4th ed. Nordic Committee on Food Analysis, Oslo, Norway.

NMKL (2005). *E. coli* Determination in Foods. Method No. 125, Nordic Committee on Food Analysis Method. (2005). Oslo, Norway.

NMKL (2005). Enterobacteriaceae Counts in Foods. Method No. 144, 3rd ed. Nordic Committee on Food Analysis Method. Oslo, Norway.

NMKL (2010). *Bacillus cereus* Determination in Foods. Method No. 67, Nordic Committee on Food Analysis Method. Oslo, Norway.

Nout, M. J. R. (1992). Upgrading traditional biotechnological processes. Report of an ad hoc panel of the board on science and technology for international development. Applications biotechnology to traditional fermented foods. National Academy Press, Washington, D. C. 11-19.

Nout, M. J. R., & Ngoddy, P. O. (1997). Technological aspects of preparing affordable fermented complementary foods. *Food Control*, *8*(5-6), 279-287.

Nout, M. R., & Rombouts, F. M. (2001). Fermented foods and their production. *Fermentation and Food Safety*, *1*, 1-19.

Nouws, S., Bogaerts, B., Verhaegen, B., Denayer, S., Piérard, D., Marchal, K., Roosens, N.H., Vanneste, K. & De Keersmaecker, S.C. (2020). Impact of DNA extraction on whole genome

sequencing analysis for characterisation and relatedness of Shiga toxin-producing Escherichia coli isolates. *Scientific Reports*, *10*(1), 1-16.

Nwachukwu, E., Achi, O. K., & Ijeoma, I. O. (2010). Lactic acid bacteria in fermentation of cereals for the production of indigenous Nigerian foods. *African Journal of Food Science and Technology*, *1*(2), 021-026.

Nyanga, L. K., Nout, M. J., Gadaga, T. H., Theelen, B., Boekhout, T., & Zwietering, M. H. (2007). Yeasts and lactic acid bacteria microbiota from masau (Ziziphus mauritiana) fruits and their fermented fruit pulp in Zimbabwe. *International Journal of Food Microbiology*, *120*(1-2), 159-166.

O'sullivan, L., Ross, R. P., & Hill, C. (2002). Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. *Biochimie*, *84*(5-6), 593-604.

Obilana, A. B. (2003). Overview: importance of millets in Africa. *World (all cultivated millet species)*, *38*, 28.

Obinna-Echem, P. C., Kuri, V., & Beal, J. (2014). Evaluation of the microbial community, acidity and proximate composition of akamu, a fermented maize food. *Journal of the Science of Food and Agriculture*, *94*(2), 331-340.

Obiri-Danso, K. (1994). Microbiological studies on corn dough fermentation. *Cereal Chemistry*, *71*(2), 186-188.

Odunfa, S. A. (1985). African fermented foods. In. BJB Wood, ed. *Microbiology of Foods*, 155-191.

Odunfa, S. A., & Oyewole, O. B. (1998). African fermented foods. In *Microbiology of Fermented Foods* (pp. 713-752). Springer, Boston, MA.

Ogunbanwo, S. T., Enitan, A. M., Emeya, P., & Okanlawon, B. M. (2005). Influence of lactic acid bacteria on fungal growth and aflatoxin production in ogi, an indigenous fermented food. *Advances in Food Sciences*, *27*(4), 189-194.

Ogunremi, O. R., Banwo, K., & Sanni, A. I. (2017). Starter-culture to improve the quality of cereal-based fermented foods: trends in selection and application. *Current Opinion in Food Science*, *13*, 38-43.

Ogunremi, O. R., Sanni, A. I., & Agrawal, R. J. J. O. A. M. (2015). Probiotic potentials of yeasts isolated from some cereal-based N igerian traditional fermented food products. *Journal of Applied Microbiology*, *119*(3), 797-808.

Oguntoyinbo, F. A. (2014). Safety challenges associated with traditional foods of West Africa. *Food Reviews International*, *30*(4), 338-358.

Oguntoyinbo, F. A., & Narbad, A. (2012). Molecular characterisation of lactic acid bacteria and in situ amylase expression during traditional fermentation of cereal foods. *Food Microbiology*, *31*(2), 254-262.

Oguntoyinbo, F. A., & Narbad, A. (2015). Multifunctional properties of Lactobacillus plantarum strains isolated from fermented cereal foods. *Journal of Functional Foods*, *17*, 621-631.

Oguntoyinbo, F. A., Tourlomousis, P., Gasson, M. J., & Narbad, A. (2011). Analysis of bacterial communities of traditional fermented West African cereal foods using culture independent methods. *International Journal of Food Microbiology*, *145*(1), 205-210.

Oh, Y. J., & Jung, D. S. (2015). Evaluation of probiotic properties of *Lactobacillus* and *Pediococcus* strains isolated from Omegisool, a traditionally fermented millet alcoholic beverage in Korea. *LWT-Food Science and Technology*, *63*(1), 437-444.

Ohashi, Y., & Ushida, K. (2009). Health-beneficial effects of probiotics: Its mode of action. *Animal Science Journal*, *80*(4), 361-371.

Ojokoh, A. O., Fayemi, O. E., Ocloo, F. C. K., & Nwokolo, F. I. (2015). Effect of fermentation on proximate composition, physicochemical and microbial characteristics of pearl millet (Pennisetum glaucum (L.) R. Br.) and Acha (Digitaria exilis (Kippist) Stapf) flour blends. *Journal of Agricultural Biotechnology and Sustainable Development*, *7*(1), 1-8.

Okeke, C.A., Ezekiel, C.N., Nwangburuka, C.C., Sulyok, M., Ezeamagu, C.O., Adeleke, R.A., Dike, S.K. & Krska, R. (2015). Bacterial diversity and mycotoxin reduction during maize fermentation (steeping) for ogi production. *Frontiers in Microbiology*, *6*, 1402.

Okereke, H. C., Achi, O. K., Ekwenye, U. N., & Orji, F. A. (2012). Antimicrobial properties of probiotic bacteria from various sources. *African Journal of Biotechnology*, *11*(39), 9416-9421.

Okoli, E. C., & Adeyemi, I. A. (1989). Manufacturing of ogi from malted (germinated) corn (Zea mays): Evaluation of chemical, pasting and sensory properties. *Journal of Food Science*, *54*(4), 971-973.

Olasupo, N. A., Schillinger, U., Narbad, A., Dodd, H., & Holzapfel, W. H. (1999). Occurrence of nisin Z production in Lactococcus lactis BFE 1500 isolated from wara, a traditional Nigerian cheese product. *International Journal of Food Microbiology*, *53*(2-3), 141-152.

Oleksy, M., & Klewicka, E. (2018). Exopolysaccharides produced by Lactobacillus sp.: Biosynthesis and applications. *Critical Reviews in Food Science and Nutrition*, *58*(3), 450-462.

Olojede, A. O., Sanni, A. I., Banwo, K., & Adesulu-Dahunsi, A. T. (2020). Sensory and antioxidant properties and in-vitro digestibility of gluten-free sourdough made with selected starter cultures. *LWT*, *129*, 109576.

Olsen, A., Halm, M., & Jakobsen, M. (1995). The antimicrobial activity of lactic acid bacteria from fermented maize (kenkey) and their interactions during fermentation. *Journal of Applied Bacteriology*, *79*(5), 506-512.

Oluwajoba, S. O., Akinyosoye, F. A., & Oyetayo, V. O. (2013). In vitro screening and selection of probiotic lactic acid bacteria isolated from spontaneously fermenting kunu-zaki. *Advances in Microbiology*, *3*(04), 309.

Omar, N. B., & Ampe, F. (2000). Microbial community dynamics during production of the Mexican fermented maize dough pozol. *Applied and Environmental Microbiology*, *66*(9), 3664-3673.

Omemu, A. M., & Faniran, O. W. (2011). Assessment of the antimicrobial activity of lactic acid bacteria isolated from two fermented maize products-ogi and kunnu-zaki. *Malaysian Journal of Microbiology*, *7*(3), 124-128.

Omemu, A. M., Oyewole, O. B., & Bankole, M. O. (2007). Significance of yeasts in the fermentation of maize for ogi production. *Food Microbiology*, *24*(6), 571-576.

Onilude, A. A., Fagade, O. E., Bello, M. M., & Fadahunsi, I. F. (2005). Inhibition of aflatoxin-producing aspergilli by lactic acid bacteria isolates from indigenously fermented cereal gruels. *African Journal of Biotechnology*, *4*(12).

Onuoha, E. C., Orukotan, A. A., & Ameh, J. B. (2017). Effect of fermentation (natural and starter) on the physicochemical, anti-nutritional and proximate composition of pearl millet used for flour production. *American Journal of Bioscience and Bioengineering*, *5*(1), 12-16.

Onyango, C., Okoth, M. W., & Mbugua, S. K. (2000). Effect of drying lactic fermented uji (an East African sour porridge) on some carboxylic acids. *Journal of the Science of Food and Agriculture*, *80*(13), 1854-1858.

Oranusi, S., Nwankwo, U. E., Onu-Okpara, I., & Olopade, B. K. (2017). Assessment of micro flora, deoxynivalenol (Don) and fumonisin contamination of grains sold in local markets, Nigeria. *Covenant Journal of Physical and Life Sciences*, *4*(2).

Osmanağaoğlu, Ö., Beyatli, Y., & Gündüz, U. (2001). Isolation and characterisation of pediocin producing Pediococcus pentosaceus Pep1 from vacuum-packed sausages. *Turkish Journal of Biology*, *25*(2), 133-143.

Ouattara, C. A. T., KounbÃ, M., moyen, R., & Traore, A. S. (2015). Isolation and identification of lactic acid and non-acid lactic bacteria from dgu of Western Africa traditional fermented millet-based food. *African Journal of Microbiology Research*, *9*(36), 2001-2005.

Owens, J. D., Allagheny, N., Kipping, G., & Ames, J. M. (1997). Formation of volatile compounds during Bacillus subtilis fermentation of soya beans. *Journal of the Science of Food and Agriculture*, *74*(1), 132-140.

Owusu-Kwarteng, J., & Akabanda, F. (2013). Applicability of nixtamalization in the processing of millet-based maasa, a fermented food in Ghana. *Journal of Food Research*, *2*(1), 59-65.

Owusu-Kwarteng, J., Akabanda, F., Nielsen, D. S., Tano-Debrah, K., Glover, R. L., & Jespersen, L. (2012). Identification of lactic acid bacteria isolated during traditional fura processing in Ghana. *Food Microbiology*, *32*(1), 72-78.

Owusu-Kwarteng, J., Tano-Debrah, K., Akabanda, F., & Jespersen, L. (2015). Technological properties and probiotic potential of Lactobacillus fermentum strains isolated from West African fermented millet dough. *BMC Microbiology*, *15*(1), 1-10.

Owusu-Kwarteng, J., Tano-Debrah, K., Glover, R. L. K., & Akabanda, F. (2010). Process characteristics and microbiology of fura produced in Ghana. *Nature and Science*, *8*(8), 41-51.

Oyelana, O. A., & Coker, A. A. (2012). Microbial contamination at different stages of production of ogi in Mowe: A rural community, Southwest, Nigeria. *Bacteriology Journal*, *2*(1), 1-11.

Oyewole, O. B. (2001). Characteristics and significance of yeasts' involvement in cassava fermentation for 'fufu'production. *International Journal of Food Microbiology*, *65*(3), 213-218.

Pandey, K. R., Naik, S. R., & Vakil, B. V. (2015). Probiotics, prebiotics and synbiotics-a review. *Journal of Food Science and Technology*, *52*(12), 7577-7587.

Panzer, K., Yilmaz, P., Weiß, M., Reich, L., Richter, M., Wiese, J., Schmaljohann, R., Labes, A., Imhoff, J.F., Glöckner, F.O. & Reich, M. (2015). Identification of habitat-specific biomes of aquatic fungal communities using a comprehensive nearly full-length 18S rRNA dataset enriched with contextual data. *PLoS One*, *10*(7), e0134377.

Park, D. H. (2018). Effects of carbon dioxide on metabolite production and bacterial communities during kimchi fermentation. *Bioscience, Biotechnology, and Biochemistry*, *82*(7), 1234-1242.

Parker, M., Zobrist, S., Donahue, C., Edick, C., Mansen, K., Hassan Zade Nadjari, M., Heerikhuisen, M., Sybesma, W., Molenaar, D., Diallo, A.M. & Milani, P. (2018). Naturally fermented milk from northern Senegal: Bacterial community composition and probiotic enrichment with *Lactobacillus rhamnosus*. *Frontiers in Microbiology*, *9*, 2218.

Paulová, L., Patáková, P., & Brányik, T. (2013). Advanced fermentation processes. *Engineering Aspects of Food Biotechnology*, 89-110.

Pearson's composition and analysis of foods. (1995). 9th Ed.

Pedersen, L. L., Owusu-Kwarteng, J., Thorsen, L., & Jespersen, L. (2012). Biodiversity and probiotic potential of yeasts isolated from Fura, a West African spontaneously fermented cereal. *International Journal of Food Microbiology*, *159*(2), 144-151.

Peña, A. S. (2007). Intestinal flora, probiotics, prebiotics, synbiotics and novel foods. *Revista Española de Enfermedades Digestivas*, *99*(11), 653.

Perez, R. H., Zendo, T., & Sonomoto, K. (2014). Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications. *Microbial Cell Factories*, *13*(1), 1-13.

Peterson, D., Bonham, K. S., Rowland, S., Pattanayak, C. W., Klepac-Ceraj, V., & Resonance Consortium. (2021). Comparative analysis of 16S rRNA gene and metagenome sequencing in pediatric gut microbiomes. *Frontiers in Microbiology*, *1651*

Petrova, P., & Petrov, K. (2020). Lactic acid fermentation of cereals and pseudocereals: Ancient nutritional biotechnologies with modern applications. *Nutrients*, *12*(4), 1118.

Peyer, L. C., Zannini, E., & Arendt, E. K. (2016). Lactic acid bacteria as sensory biomodulators for fermented cereal-based beverages. *Trends in Food Science & Technology*, *54*, 17-25.

Pfeiler, E. A., & Klaenhammer, T. R. (2007). The genomics of lactic acid bacteria. *Trends in Microbiology*, *15*(12), 546-553.

Piard, J. C., & Desmazeaud, M. (1991). Inhibiting factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end-products. *Le lait*, *71*(5), 525-541.

Pidoux, M., Marshall, V. M., Zanoni, P., & Brooker, B. (1990). Lactobacilli isolated from sugary kefir grains capable of polysaccharide production and minicell formation. *Journal of Applied Bacteriology*, *69*(3), 311-320.

Plumridge, A., Hesse, S. J., Watson, A. J., Lowe, K. C., Stratford, M., & Archer, D. B. (2004). The weak acid preservative sorbic acid inhibits conidial germination and mycelial growth of Aspergillus niger through intracellular acidification. *Applied and Environmental Microbiology*, *70*(6), 3506-3511.

Poitrenaud, B. (2003). Commercial starters in France. In *Handbook of Dough Fermentations* (pp. 217-250). CRC Press.

Poutanen, K. S. (2020). Cereal raw material pretreatment. In *Breakfast Cereals and How They Are Made* (pp. 97-107). AACC International Press.

Poutanen, K., Flander, L., & Katina, K. (2009). Sourdough and cereal fermentation in a nutritional perspective. *Food Microbiology*, *26*(7), 693-699.

Prasad, J., Gill, H., Smart, J., & Gopal, P. K. (1998). Selection and characterisation of *Lactobacillus* and *Bifidobacterium* strains for use as probiotics. *International Dairy Journal*, *8*(12), 993-1002.

Price, R. L., & Jorgensen, K. V. (1985). Effects of processing on aflatoxin levels and on mutagenic potential of tortillas made from naturally contaminated corn. *Journal of Food Science*, *50*(2), 347-349.

Pridmore, R. D., Pittet, A. C., Praplan, F., & Cavadini, C. (2008). Hydrogen peroxide production by *Lactobacillus johnsonii* NCC 533 and its role in anti-Salmonella activity. *FEMS Microbiology Letters*, *283*(2), 210-215.

Psani, M., & Kotzekidou, P. (2006). Technological characteristics of yeast strains and their potential as starter adjuncts in Greek-style black olive fermentation. *World Journal of Microbiology and Biotechnology*, *22*(12), 1329-1336.

Psomas, E., Andrighetto, C., Litopoulou-Tzanetaki, E., Lombardi, A., & Tzanetakis, N. (2001). Some probiotic properties of yeast isolates from infant faeces and Feta cheese. *International Journal of Food Microbiology*, *69*(1-2), 125-133.

Pswarayi, F., & Gänzle, M. G. (2019). Composition and origin of the fermentation microbiota of mahewu, a Zimbabwean fermented cereal beverage. *Applied and Environmental Microbiology*, *85*(11), e03130-18.

Qin, H., Sun, Q., Pan, X., Qiao, Z., & Yang, H. (2016). Microbial diversity and biochemical analysis of Suanzhou: a traditional Chinese fermented cereal gruel. *Frontiers in Microbiology*, *7*, 1311.

Ragaee, S., Abdel-Aal, E. S. M., & Noaman, M. (2006). Antioxidant activity and nutrient composition of selected cereals for food use. *Food Chemistry*, *98*(1), 32-38.

Rahaman, T., Vasiljevic, T., & Ramchandran, L. (2016). Effect of processing on conformational changes of food proteins related to allergenicity. *Trends in Food Science & Technology*, *49*, 24-34.

Rajkowska, K., & Kunicka-Styczyńska, A. (2010). Probiotic properties of yeasts isolated from chicken feces and kefirs. *Polish Journal of Microbiology*, *59*(4), 257-263.

Rantsiou, K., & Cocolin, L. (2006). New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: a review. *International Journal of Food Microbiology*, *108*(2), 255-267.

Rattanachaikunsopon, P., & Phumkhachorn, P. (2010). Lactic acid bacteria: their antimicrobial compounds and their uses in food production. *Annals of Biological Research*, *1*(4), 218-228.

Ray, M., Ghosh, K., Singh, S., & Mondal, K. C. (2016). Folk to functional: an explorative overview of rice-based fermented foods and beverages in India. *Journal of Ethnic Foods*, *3*(1), 5-18.

Reddy, K. R. N., Salleh, B., Saad, B., Abbas, H. K., Abel, C. A., & Shier, W. T. (2010). An overview of mycotoxin contamination in foods and its implications for human health. *Toxin Reviews*, *29*(1), 3-26.

Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Escámez, P. S. F. & Robertson, L. (2018). Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 8: suitability of taxonomic units notified to EFSA until March 2018. *Efsa Journal*, *16(1)*, *e05131*.

Riesenfeld, C. S., Schloss, P. D., & Handelsman, J. (2004). Metagenomics: genomic analysis of microbial communities. *Annual review of genetics*, *38*, 525-552.

Rizzo, J. M., & Buck, M. J. (2012). Key principles and clinical applications of "next-generation" DNA sequencing. *Cancer Prevention Research*, *5*(7), 887-900.

Rochfort, S., Ezernieks, V., Bastian, S. E., & Downey, M. O. (2010). Sensory attributes of wine influenced by variety and berry shading discriminated by NMR metabolomics. *Food Chemistry*, *121*(4), 1296-1304.

Rodríguez, H., Curiel, J. A., Landete, J. M., de las Rivas, B., de Felipe, F. L., Gómez-Cordovés, C., ... & Muñoz, R. (2009). Food phenolics and lactic acid bacteria. *International Journal of Food Microbiology*, *132*(2-3), 79-90.

Rodriguez-Brito, B., Rohwer, F., & Edwards, R. A. (2006). An application of statistics to comparative metagenomics. *BMC Bioinformatics*, *7*(1), 1-11.

Ross, A. B., Zangger, A., & Guiraud, S. P. (2014). Cereal foods are the major source of betaine in the Western diet–analysis of betaine and free choline in cereal foods and updated assessments of betaine intake. *Food Chemistry*, *145*, 859-865.

Ross, R. P., Morgan, S., & Hill, C. (2002). Preservation and fermentation: past, present and future. *International Journal of Food Microbiology*, *79*(1-2), 3-16.

Roudil, L., Russo, P., Berbegal, C., Albertin, W., Spano, G., & Capozzi, V. (2020). Non-Saccharomyces commercial starter cultures: scientific trends, recent patents and innovation in the wine sector. *Recent Patents on Food, Nutrition & Agriculture*, *11*(1), 27-39.

Ruas-Madiedo, P., & De Los Reyes-Gavilán, C. G. (2005). Invited review: methods for the screening, isolation, and characterisation of exopolysaccharides produced by lactic acid bacteria. *Journal of Dairy Science*, *88*(3), 843-856.

Ruas-Madiedo, P., Hugenholtz, J., & Zoon, P. (2002). An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. *International Dairy Journal*, *12*(2-3), 163-171.

Russo, P., Arena, M. P., Fiocco, D., Capozzi, V., Drider, D., & Spano, G. (2017). Lactobacillus plantarum with broad antifungal activity: A promising approach to increase safety and shelf-life of cereal-based products. *International Journal of Food Microbiology*, *247*, 48-54.

Sabillón, L., Stratton, J., Rose, D., & Bianchini, A. (2021). Microbiological survey of equipment and wheat-milled fractions of a milling operation. *Cereal Chemistry*, *98*(1), 44-51.

Sade, F. O. (2009). Proximate, antinutritional factors and functional properties of processed pearl millet (Pennisetum glaucum). *Journal of Food Technology*, *7*(3), 92-97.

Saeed, M., Khan, W. A., Shabbir, M. A., Khan, M. I., Randhawa, M. A., & Yasmin, I. (2014). Bacteriocins as a natural antimicrobial agent in food preservation: A review. *Pakistan Journal of Food Sciences*, *24*(4), 244-255.

Saleh, A. S., Zhang, Q., Chen, J., & Shen, Q. (2013). Millet grains: nutritional quality, processing, and potential health benefits. *Comprehensive Reviews in Food Science and Food Safety*, *12*(3), 281-295.

Salmerón, I., Rozada, R., Thomas, K., Ortega-Rivas, E., & Pandiella, S. S. (2014). Sensory characteristics and volatile composition of a cereal beverage fermented with Bifidobacterium breve NCIMB 702257. *Food Science and Technology International*, *20*(3), 205-213.

Salmerón, I., Thomas, K., & Pandiella, S. S. (2015). Effect of potentially probiotic lactic acid bacteria on the physicochemical composition and acceptance of fermented cereal beverages. *Journal of Functional Foods*, *15*, 106-115.

Samtiya, M., Aluko, R. E., & Dhewa, T. (2020). Plant food anti-nutritional factors and their reduction strategies: An overview. *Food Production, Processing and Nutrition*, *2*(1), 1-14.

Sanalibaba, P., & Çakmak, G. A. (2016). Exopolysaccharides production by lactic acid bacteria. *Applied Microbiology. Open Access*, *2(115), 10-4172*.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the national academy of sciences*, *74*(12), 5463-5467.

Sanni, A. I., Morlon-Guyot, J., & Guyot, J. P. (2002). New efficient amylase-producing strains of *Lactobacillus plantarum* and *L. fermentum* isolated from different Nigerian traditional fermented foods. *International Journal of Food Microbiology*, *72*(1-2), 53-62.

Sass, P., Jansen, A., Szekat, C., Sass, V., Sahl, H. G., & Bierbaum, G. (2008). The lantibiotic mersacidin is a strong inducer of the cell wall stress response of *Staphylococcus aureus*. *BMC Microbiology*, *8*(1), 1-11.

Savadogo, A., Ouattara, A. C., Bassole, H. I., & Traore, S. A. (2006). Bacteriocins and lactic acid bacteria-a minireview. *African Journal of Biotechnology*, *5*(9), 678–683.

Sawadogo-Lingani, H., Lei, V., Diawara, B., Nielsen, D. S., Møller, P. L., Traoré, A. S., & Jakobsen, M. (2007). The biodiversity of predominant lactic acid bacteria in dolo and pito wort for the production of sorghum beer. *Journal of Applied Microbiology*, *103*(4), 765-777.

Schadt, E. E., Turner, S., & Kasarskis, A. (2010). A window into third-generation sequencing. *Human Molecular Genetics*, *19*(R2), R227-R240.

Schillinger, U., & Lücke, F. K. (1989). Einsatz von milchsäurebakterien als schutzkulturen bei fleischerzeugnissen. *Fleischwirtschaft*, *69*(10), 1581-1586.

Schnürer, J., & Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science & Technology*, *16*(1-3), 70-78.

Scott, E., & Bloomfield, S. F. (1990). The survival and transfer of microbial contamination via cloths, hands and utensils. *Journal of Applied Bacteriology*, *68*(3), 271-278.

Scott, R., & Sullivan, W. C. (2008). Ecology of fermented foods. *Human Ecology Review*, 25-31.

Sekwati-Monang, B. (2011). Microbiological and chemical characterisation of ting, a sorghum-based gluten-free fermented cereal product from Botswana. Doctoral thesis, University of Alberta.

Sekwati-Monang, B., Valcheva, R., & Gänzle, M. G. (2012). Microbial ecology of sorghum sourdoughs: effect of substrate supply and phenolic compounds on composition of fermentation microbiota. *International Journal of Food Microbiology*, *159*(3), 240-246.

Serratì, S., De Summa, S., Pilato, B., Petriella, D., Lacalamita, R., Tommasi, S., & Pinto, R. (2016). Next-generation sequencing: advances and applications in cancer diagnosis. *OncoTargets and Therapy*, *9*, 7355.

Shah, N. P. (2000). Probiotic bacteria: selective enumeration and survival in dairy foods. *Journal of Dairy Science*, *83*(4), 894-907.

Shangpliang, H. N. J., Rai, R., Keisam, S., Jeyaram, K., & Tamang, J. P. (2018). Bacterial community in naturally fermented milk products of Arunachal Pradesh and Sikkim of India analysed by high-throughput amplicon sequencing. *Scientific Reports*, *8*(1), 1-10.

Sharma, A., & Kapoor, A. C. (1996). Levels of antinutritional factors in pearl millet as affected by processing treatments and various types of fermentation. *Plant Foods for Human Nutrition*, *49*(3), 241-252.

Sharpe, M. E. (1981). The genus Lactobacillus, in the Procaryotes: A handbook on Habitats, Isolation and Identification of Bacteria. Eds. Starr MP, Stolp H, Trüper HG, Balows A. Schlegel HG. pp. 1653-1679. Berlin: Springer-Verlag.

Shetty, P. H., & Jespersen, L. (2006). *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents. *Trends in Food Science & Technology*, *17*(2), 48-55.

Shetty, P. H., Hald, B., & Jespersen, L. (2007). Surface binding of aflatoxin B1 by *Saccharomyces cerevisiae* strains with potential decontaminating abilities in indigenous fermented foods. *International Journal of Food Microbiology*, *113*(1), 41-46.

Siezen, R. J., van Enckevort, F. H., Kleerebezem, M., & Teusink, B. (2004). Genome data mining of lactic acid bacteria: the impact of bioinformatics. *Current Opinion in Biotechnology*, *15*(2), 105-115.

Sim, K. Y., Yee, C. F., & Anton, A. (2012). Probiotic potential and antimicrobial activities of micro-organisms isolated from an indigenous fish sauce. *Borneo Science*. *31*, 57-63

Simwaka, J. E., Chamba, M. V. M., Huiming, Z., Masamba, K. G., & Luo, Y. (2017). Effect of fermentation on physicochemical and antinutritional factors of complementary foods from millet, sorghum, pumpkin and amaranth seed flours. *International Food Research Journal*, *24*(5).

Sindhu, S. C., & Khetarpaul, N. (2001). Probiotic fermentation of indigenous food mixture: effect on antinutrients and digestibility of starch and protein. *Journal of Food Composition and Analysis*, *14*(6), 601-609.

Singh, A. K., Rehal, J., Kaur, A., & Jyot, G. (2015). Enhancement of attributes of cereals by germination and fermentation: a review. *Critical Reviews in Food Science and Nutrition*, *55*(11), 1575-1589.

Siragusa, S., Di Cagno, R., Ercolini, D., Minervini, F., Gobbetti, M., & De Angelis, M. (2009). Taxonomic structure and monitoring of the dominant population of lactic acid bacteria during wheat flour sourdough type I propagation using *Lactobacillus sanfranciscensis* starters. *Applied and Environmental Microbiology*, *75*(4), 1099-1109.

Sivamaruthi, B. S., Kesika, P., & Chaiyasut, C. (2019). Toxins in fermented foods: prevalence and preventions—a mini review. *Toxins*, *11*(1), 4.

Sleator, R. D., Shortall, C., & Hill, C. (2008). Metagenomics. *Letters in Applied Microbiology*, *47*(5), 361-366.

Smith, H. B., Dal Grande, F., Muggia, L., Keuler, R., Divakar, P. K., Grewe, F., ... & Leavitt, S. D. (2020). Metagenomic data reveal diverse fungal and algal communities associated with the lichen symbiosis. *Symbiosis*, *82*(1), 133-147.

Sniegowski, P. D., Dombrowski, P. G., & Fingerman, E. (2002). Saccharomyces cerevisiae and Saccharomyces paradoxus coexist in a natural woodland site in North America and display different levels of reproductive isolation from European conspecifics. *FEMS Yeast Research*, *1*(4), 299-306.

Sokrab, A. M., Mohamed Ahmed, I. A., & Babiker, E. E. (2012). Effect of malting and fermentation on antinutrients, and total and extractable minerals of high and low phytate corn genotypes. *International Journal of Food Science & Technology*, *47*(5), 1037-1043.

Solieri, L., Dakal, T. C., & Giudici, P. (2013). Next-generation sequencing and its potential impact on food microbial genomics. *Annals of Microbiology*, *63*(1), 21-37.

Songré-Ouattara, L. T., Mouquet-Rivier, C., Icard-Vernière, C., Humblot, C., Diawara, B., & Guyot, J. P. (2008). Enzyme activities of lactic acid bacteria from a pearl millet fermented gruel (ben-saalga) of functional interest in nutrition. *International Journal of Food Microbiology*, *128*(2), 395-400.

Songré-Ouattara, L. T., Mouquet-Rivier, C., Icard-Vernière, C., Rochette, I., Diawara, B., & Guyot, J. P. (2009). Potential of amylolytic lactic acid bacteria to replace the use of malt for partial starch hydrolysis to produce African fermented pearl millet gruel fortified with groundnut. *International Journal of Food Microbiology*, *130*(3), 258-264.

Soomro, A. H., Masud, T., & Anwaar, K. (2002). Role of lactic acid bacteria (LAB) in food preservation and human health-a review. *Pakistan Journal of Nutrition*, *1*(1), 20-24.

Soro-Yao, A. A., Kouakou Brou, G. A., Thonart, P., & Djè, K. M. (2014). The use of lactic acid bacteria starter cultures during the processing of fermented cereal-based foods in West Africa: a review. *Tropical Life Sciences Research*, *25*(2), 81.

Sripriya, G., Antony, U., & Chandra, T. S. (1997). Changes in carbohydrate, free amino acids, organic acids, phytate and HCl extractability of minerals during germination and fermentation of finger millet (Eleusine coracana). *Food Chemistry*, *58*(4), 345-350.

Stam, H., Hoogland, M., & Laane, C. (1998). Food flavours from yeast. In *Microbiology of Fermented Foods* (pp. 505-542). Springer, Boston, MA.

Stanbury, P. F., Whitaker, A., & Hall, S. J. (2013). *Principles of Fermentation Technology*. Elsevier.

Starkutė, V., Krunglevičiūtė, V., Bartkienė, E., Bartkevičs, V., Žadeikė, D., & Juodeikienė, G. (2017). Cereal by-products conversion to stock with high content of *P. acidilactici* by using enzymatic treatment combined with solid state and submerged fermentation. In *Foodbalt 2017: 11th Baltic Conference on Food Science and Technology" Food science and technology in a changing world", Jelgava, April 27-28, 2017: abstract book/Latvia University of Agriculture Faculty of Food Technology. Sl: sn, 2017, 2017*.

Statistics, Research and Information (SRID). (2016). Agriculture in Ghana: facts and figures. Statistical, Research and Information Directorate, Ministry of Food and Agriculture (MoFa) Ghana.

Steiner, P., & Sauer, U. (2003). Long-term continuous evolution of acetate resistant Acetobacter aceti. *Biotechnology and bioengineering*, *84*(1), 40-44.

Steinkraus, K. H. (1983). Fermented foods, feeds and beverages. *Biotechnology Advances*, *1*(1), 31-46.

Steinkraus, K. H. (1997). Classification of fermented foods: worldwide review of household fermentation techniques. *Food Control*, *8*(5-6), 311-317.

Steinkraus, K. H. (2002). Fermentations in world food processing. *Comprehensive Reviews in Food Science and Food Safety*, *1*(1), 23-32.

Steinkraus, K.H., (1996). Handbook of indigenous fermented foods, (2nd Ed.). *Marcel Dekker Inc. New York.* (No. 660.28449 S83 1996).

Stroka, J., & Anklam, E. (1991). Quantitative analysis for aflatoxins. *JAOAC, 74*, 81-4.

Sulieman, A. M. (2017). *Microbial Starter Cultures*. Lap Lambert Academic Publishing. Beau Bassin, Mauritius, ISBN: 978-620-2-05961-9.

Sulmiyati, S., Said, N. S., Fahrodi, D. U., Malaka, R., & Maruddin, F. (2019). The Characteristics Yeast Isolated from Commercial Kefir Grain, Indonesia. *Hasanuddin Journal of Animal Science (HAJAS)*, *1*(1), 26-36.

Šušković, J., Kos, B., Beganović, J., Leboš Pavunc, A., Habjanič, K., & Matošić, S. (2010). Antimicrobial activity–the most important property of probiotic and starter lactic acid bacteria. *Food Technology and Biotechnology*, *48*(3), 296-307.

Sweeney, J. Y., Kuehne, H. A., & Sniegowski, P. D. (2004). Sympatric natural Saccharomyces cerevisiae and S. paradoxus populations have different thermal growth profiles. *FEMS Yeast Research*, *4*(4-5), 521-525.

Syal, P., & Vohra, A. (2013). Probiotic potential of yeasts isolated from traditional Indian fermented foods. *International Journal of Microbiology Research*, *5*(2), 390-398.

Tamang, J. P. (2010). Diversity of fermented foods. In *Fermented foods and Beverages of the World* (pp. 53-96). CRC Press.

Tamang, J. P., Shin, D. H., Jung, S. J., & Chae, S. W. (2016). Functional properties of microorganisms in fermented foods. *Frontiers in Microbiology*, *7*, 578.

Taylor, J. R. N., Barrion, S. C., & Rooney, L. W. (2010). Pearl millet-new developments in ancient food grain. *Cereal Foods World*, *55*(1), 16-19.

Teniola, O. D., & Odunfa, S. A. (2001). The effects of processing methods on the levels of lysine, methionine and the general acceptability of ogi processed using starter cultures. *International Journal of Food Microbiology*, *63*(1-2), 1-9.

Terzi, V., Tumino, G., Stanca, A. M., & Morcia, C. (2014). Reducing the incidence of cereal head infection and mycotoxins in small grain cereal species. *Journal of Cereal Science*, *59*(3), 284-293.

Theron, M. M., & Lues, J. F. (2007). Organic acids and meat preservation: a review. *Food Reviews International*, *23*(2), 141-158.

Theron, M. M., & Lues, J. R. (2010). *Organic acids and food preservation*. CRC press.

Toldr´a, F. (2004). Handbook of fermented meats and poultry. In: Smith JS, Hui YH, editors. Food processing: principles and applications, Chapter 23, Ames, IA: Blackwell Publ 399–415.

Toma, M. M., & Pokrotnieks, J. (2006). Probiotics as functional food: microbiological and medical aspects. *Acta Universitatis Latviensis*, *710*, 117-129.

Torino, M. I., Taranto, M. P., & De Valdez, G. F. (2005). Citrate catabolism and production of acetate and succinate by Lactobacillus helveticus ATCC 15807. *Applied microbiology and biotechnology*, *69*(1), 79-85.

Torriani, S., Felis, G. E., & Dellaglio, F. (2001). Differentiation of Lactobacillus plantarum, L. pentosus, and L. paraplantarum by recA gene sequence analysis and multiplex PCR assay with recA gene-derived primers. *Applied and Environmental Microbiology*, *67*(8), 3450-3454.

Totté, A., Tine, E., Seye, N., Mathiam, J. M., Roblain, D., & Thonard, P. (2003). Innovation et transfert de technologie: Cas du contrôle de la fermentation du mil par l′ utilisation d′ un starter lactique. In *Proceedings of the 2nd International workshop in Food-based Approaches for a Healthy Nutrition. Ouagadougou, Burkina Faso* (pp. 23-28).

Tsafrakidou, P., Michaelidou, A. M., & G Biliaderis, C. (2020). Fermented cereal-based products: Nutritional aspects, possible impact on gut microbiota and health implications. *Foods*, *9*(6), 734

Tsai, I. J., Bensasson, D., Burt, A., & Koufopanou, V. (2008). Population genomics of the wild yeast Saccharomyces paradoxus: Quantifying the life cycle. *Proceedings of the National Academy of Sciences*, *105*(12), 4957-4962.

Ubbor, S. C., & Akobundu, E. N. T. (2009). Quality characteristics of cookies from composite flours of watermelon seed, cassava and wheat. *Pakistan Journal of Nutrition*, *8*(7), 1097-1102.

Ugliano, M., Squillante, E., Genovese, A., & Moio, L. (2003). Investigation on aroma compounds of Modena balsamic vinegars. In *Flavour Research at the Dawn of the Twenty-first Century-Proceedings of the 10th Weurman Flavour Research Symposium, Beaune, France, 25-28 June, 2002* (pp. 733-736).

Valerio, F., Favilla, M., De Bellis, P., Sisto, A., de Candia, S., & Lavermicocca, P. (2009). Antifungal activity of strains of lactic acid bacteria isolated from a semolina ecosystem against

Penicillium roqueforti, *Aspergillus niger* and *Endomyces fibuliger* contaminating bakery products. *Systematic and Applied Microbiology*, *32*(6), 438-448.

van Heel, A. J., Montalban-Lopez, M., & Kuipers, O. P. (2011). Evaluating the feasibility of lantibiotics as an alternative therapy against bacterial infections in humans. *Expert Opinion on Drug Metabolism & Toxicology*, *7*(6), 675-680.

Varsha, K. K., & Nampoothiri, K. M. (2016). Appraisal of lactic acid bacteria as protective cultures. *Food Control*, *69*, 61-64.

Vasiljevic, T., & Shah, N. P. (2008). Probiotics—from Metchnikoff to bioactives. *International Dairy Journal*, *18*(7), 714-728.

Vaughan-Martini, A., & Martini, A. (2011). Saccharomyces meyen ex reess (1870). In *The yeasts* (pp. 733-746). Elsevier.

Vázquez-Fresno, R., Llorach, R., Urpi-Sarda, M., Khymenets, O., Bulló, M., Corella, D., ... & Andres-Lacueva, C. (2015). An NMR metabolomics approach reveals a combined-biomarkers model in a wine interventional trial with validation in free-living individuals of the PREDIMED study. *Metabolomics*, *11*(4), 797-806.

Vieira-Dalodé, G., Jespersen, L., Hounhouigan, J., Moller, P. L., Nago, C. M., & Jakobsen, M. (2007). Lactic acid bacteria and yeasts associated with gowé production from sorghum in Bénin. *Journal of Applied Microbiology*, *103*(2), 342-349.

Viel, J. H., van Tilburg, A. Y., & Kuipers, O. P. (2021). Characterisation of Leader Processing Shows That Partially Processed Mersacidin Is Activated by AprE after Export. *Frontiers in microbiology*, 3259.

Voelkerding, K. V., Dames, S. A., & Durtschi, J. D. (2009). Next-generation sequencing: from basic research to diagnostics. *Clinical Chemistry*, *55*(4), 641-658.

Vogelmann, S. A., Seitter, M., Singer, U., Brandt, M. J., & Hertel, C. (2009). Adaptability of lactic acid bacteria and yeasts to sourdoughs prepared from cereals, pseudocereals and cassava and use of competitive strains as starters. *International Journal of Food Microbiology*, *130*(3), 205-212.

Wakil, S. M., & Daodu, A. A. (2011). Physiological properties of a microbial community in spontaneous fermentation of maize (Zea mays) for ogi production. *International Research Journal of Microbiology*, *2*(3), 109-115.

Wakil, S. M., & Kazeem, M. O. (2012). Quality assessment of weaning food produced from fermented cereal-legume blends using starters. *International Food Research Journal*, *19*(4), 1679-1685.

Wakil, S. M., Laba, S. A., & Fasika, S. A. (2014). Isolation and identification of antimicrobial-producing lactic acid bacteria from fermented cucumber. *African Journal of Biotechnology*, *13*(25).

Wakil, S. M., Onilude, A. A., & Ball, A. (2008). Dynamics and diversity of bacterial communities of fermented weaning foods via denaturing gradient gel electrophoresis PCR-DGGE. *Research Journal of Microbiology*, *3*(11), 630-640.

Walter, J. (2008). Ecological role of lactobacilli in the gastrointestinal tract: implications for fundamental and biomedical research. *Applied and Environmental Microbiology*, *74*(16), 4985-4996.

Walter, J., Britton, R. A., & Roos, S. (2011). Host-microbial symbiosis in the vertebrate gastrointestinal tract and the *Lactobacillus reuteri* paradigm. *Proceedings of the National Academy of Sciences*, *108*(Supplement 1), 4645-4652.

Wang, J., & Xie, Y. (2020). Review on microbial degradation of zearalenone and aflatoxins. *Grain & Oil Science and Technology*, *3*(3), 117-125.

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, *73*(16), 5261-5267.

Wang, Y., & Qian, P. Y. (2009). Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *PloS One*, *4*(10), e7401.

Warnecke, F., & Hugenholtz, P. (2007). Building on basic metagenomics with complementary technologies. *Genome Biology*, *8*(12), 1-5.

Warringer, J., Zörgö, E., Cubillos, F.A., Zia, A., Gjuvsland, A., Simpson, J.T., Forsmark, A., Durbin, R., Omholt, S.W., Louis, E.J. & Liti, G. (2011). Trait variation in yeast is defined by population history. *PLoS Genetics*, *7*(6), e1002111.

Weckwerth, W., & Fiehn, O. (2002). Can we discover novel pathways using metabolomic analysis?. *Current Opinion in Biotechnology*, *13*(2), 156-160.

Wei, F., Furihata, K., Hu, F., Miyakawa, T., & Tanokura, M. (2010). Complex mixture analysis of organic compounds in green coffee bean extract by two-dimensional NMR spectroscopy. *Magnetic Resonance in Chemistry*, *48*(11), 857-865.

Weizman, Z., Asli, G., & Alsheikh, A. (2005). Effect of a probiotic infant formula on infections in child care centers: comparison of two probiotic agents. *Pediatrics*, *115*(1), 5-9.

Weldemichael, H., Stoll, D., Weinert, C., Berhe, T., Admassu, S., Alemu, M., & Huch, M. (2019). Characterisation of the microbiota and volatile components of kocho, a traditional fermented food of Ethiopia. *Heliyon*, *5*(6), e01842.

Welman, A. D., & Maddox, I. S. (2003). Exopolysaccharides from lactic acid bacteria: perspectives and challenges. *Trends in Biotechnology*, *21*(6), 269-274.

Whitehead, K., Versalovic, J., Roos, S., & Britton, R. A. (2008). Genomic and genetic characterisation of the bile stress response of probiotic Lactobacillus reuteri ATCC 55730. *Applied and Environmental Microbiology*, *74*(6), 1812-1819.

Wilkinson, V. M., & Gould, G. W. (1996). *Food irradiation: a reference guide*. Woodhead Publishing.

Wong, N. (2018). *Developing nanoparticulate oligonucleotides as a target-specific antimicrobial for modulation of complex gut microbiota* (Doctoral dissertation, University of East Anglia).

Wu, Q., Jezkova, A., Yuan, Z., Pavlikova, L., Dohnal, V., & Kuca, K. (2009). Biological degradation of aflatoxins. *Drug Metabolism Reviews*, *41*(1), 1-7.

Xu, Y., Zhou, T., Tang, H., Li, X., Chen, Y., Zhang, L., & Zhang, J. (2020). Probiotic potential and amylolytic properties of lactic acid bacteria isolated from Chinese fermented cereal foods. *Food Control*, *111*, 107057.

Yadav, D. N., Sharma, M., Chikara, N., Anand, T., & Bansal, S. (2014). Quality characteristics of vegetable-blended wheat–pearl millet composite pasta. *Agricultural Research*, *3*(3), 263-270.

Yagoub, S. O. (2009). Isolation of Enterobacteriaceae and Pseudomonas spp. from raw fish sold in fish market in Khartoum state. *African Journal of Bacteriology Research*, *1*(7), 085-088.

Yan, Y. Z., Qian, Y. L., Chen, J. Y., & Han, B. Z. (2013). Microbial composition during Chinese soy sauce koji-making based on culture dependent and independent methods. *Food Microbiology*, *34*(1), 189-195.

Yang, X., Wan, Z., Perry, L., Lu, H., Wang, Q., Zhao, C., Li, J., Xie, F., Yu, J., Cui, T. & Wang, T. (2012). Early millet use in northern China. *Proceedings of the National Academy of Sciences*, *109*(10), 3726-3730.

Yao, A.A., Dortu, C., Egounlety, M., Pinto, C., Edward, V.A., Huch, M., Franz, C.M., Holzapfel, W., Mbugua, S., Mengu, M. & Thonart, P. (2009). Production of freeze-dried lactic acid bacteria starter culture for cassava fermentation into gari. *African Journal of Biotechnology*, *8*(19), 4996-5004.

Young, N. W. G., & O'sullivan, G. R. (2011). The influence of ingredients on product stability and shelf life. In *Food and beverage stability and shelf life* (pp. 132-183). Woodhead Publishing.

Zacharof, M. P., & Lovitt, R. W. (2012). Bacteriocins produced by lactic acid bacteria a review article. *Apcbee Procedia*, *2*, 50-56.

Zalán, Z., Németh, E., Baráth, Á., & Halász, A. (2005). Influence of growth medium on hydrogen peroxide and bacteriocin production of *Lactobacillus strains*. *Food Technology and Biotechnology*, *43*(3), 219-225.

Zanello, G., Meurens, F., Berri, M., Chevaleyre, C., Melo, S., Auclair, E., & Salmon, H. (2011). *Saccharomyces cerevisiae* decreases inflammatory responses induced by F4+ enterotoxigenic *Escherichia coli* in porcine intestinal epithelial cells. *Veterinary Immunology and Immunopathology*, *141*(1-2), 133-138.

Zannini, E., Pontonio, E., Waters, D. M., & Arendt, E. K. (2012). Applications of microbial fermentations for production of gluten-free products and perspectives. *Applied Microbiology and Biotechnology*, *93*(2), 473-485.

Zhao, W., Chen, Y., Sun, Z., Wang, J., Zhou, Z., Sun, T., Wang, L., Chen, W. & Zhang, H. (2011). Complete genome sequence of *Lactobacillus helveticus* H10. *Journal of Bacteriology*. 2666-2667.

Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M., Harris, H.M., Mattarelli, P., O'toole, P.W., Pot, B., Vandamme, P., Walter, J. & Watanabe, K. (2020). A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *International Journal of Systematic and Evolutionary Microbiology*, *70*(4), 2782-2858.

Zhou, J., Liu, X., Jiang, H., & Dong, M. (2009). Analysis of the microflora in Tibetan kefir grains using denaturing gradient gel electrophoresis. *Food Microbiology*, *26*(8), 770-775.

Zhou, X., Ren, L., Li, Y., Zhang, M., Yu, Y., & Yu, J. (2010). The next-generation sequencing technology: a technology review and future perspective. *Science China Life Sciences*, *53*(1), 44-57.

Zoumpopoulou, G., Pot, B., Tsakalidou, E., & Papadimitriou, K. (2017). Dairy probiotics: beyond the role of promoting gut and immune health. *International Dairy Journal*, *67*, 46-60.

APPENDICES

Appendix 1

Ethical approval for the study

UNIVERSITY OF GHANA
ETHICS COMMITTEE FOR BASIC AND APPLIED SCIENCES (ECBAS
P. O. Box LG 1195, Legon-Accra

Ref. No: ECBAS 014/19-20

22nd February, 2021.

Mrs. Amy Atter
Department of Nutrition and Food Science
University of Ghana
Legon, Accra

Dear Mrs. Atter,

ECBAS 014/19-20: MICROBIOTA OF FERMENTING MILLET IN HAUSA KOKO PRODUCTION: THEIR DIVERSITY, FERMENTATIVE CHARACTERISTICS AND POTENTIAL STARTER CULTURE DEVELOPMENT

This is to inform you that the above referenced study has been presented to the Ethics Committee for Basic and Applied Sciences for a full board review and the following actions taken subject to the conditions and explanation provided below:

Expiry Date: 28/11/2021

On Agenda for: Amendment Review

Date of Submission: 29/11/2020

ECBAS Action: Approved

Reporting: Annually

Please accept my congratulations.

Yours sincerely,

Professor Daniel Bruce Sarpong
ECBAS Chairperson

THE ADMINISTRATOR
UNIVERSITY OF GHANA
LEGON

Appendix 2

Traditional *Hausa koko* processing stages



Dry millet grains | Steeping of millet (12-24 h) | Drained millet with spices



Milled millet with spice | Addition of water to milled millet | Sieving with a cheese cloth



Roughage/chaff is discarded | Removing of cheese cloth | Fermentation of slurry (8-12 h)

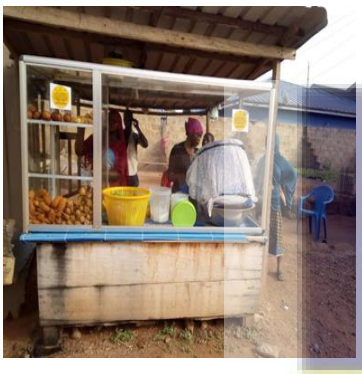
End of slurry fermentation | Removal of surface foam | Mixing of supernatant and sediment together

Boiling of water | Fetching a portion of the mixture | Mixture fetched into a container

Fetching of boiling water from the pot on fire | Addition of boiling water to the mixture | Stirring to get the desired consistency

Final product, *Hausa koko* material

Hausa koko covered with polyethylene sheets and a white lace material and tied with a rope ready to be conveyed to the point of sale



Hausa *koko* displayed for sale

Packaged *Hausa koko* in polyethylene/disposable cup/bowl (ready-to-eat)

Appendix 3

pH and microbial counts (log CFU/g) at various stages of *Hausa koko* production from 5 processors

Processors	Stages	pH	LAB (MRS)	LAB (M17)	Yeast	A. Mesophiles	Enterobact.	*S. aureus*	*B. cereus*	*E. coli*	*Salmonella* spp
Tamale	TAD-D	6.02^{a}	3.18^{c}	1.71^{c}	2.02^{d}	4.74^{d}	2.49^{b}	2.90^{a}	0	1.68^{a}	nd
	TAD-12h	4.08^{b}	7.84^{a}	3.36^{b}	5.76^{a}	6.92^{c}	2.68^{ab}	1.65^{b}	0	1.33^{b}	nd
	TAD-M	3.91^{c}	-	-	-	6.76^{c}	2.83^{a}	2.86^{a}	0	0	nd
	TAD-Su	3.27^{e}	7.76^{a}	3.27^{b}	4.92^{b}	7.88^{b}	1.67^{d}	0	0	0	nd
	TAD-Sd	3.28^{e}	7.64^{b}	3.56^{a}	4.97^{b}	8.93^{a}	1.95^{c}	0	0	0	nd
	TAD-K	3.65^{d}	2.77^{d}	1.49^{d}	2.27^{c}	3.03^{e}	0	0	0	0	nd
Sunyani	SUN-D	6.53^{a}	3.45^{d}	1.97^{c}	2.26^{e}	5.89^{e}	3.89^{a}	0	1.82^{bc}	2.72^{a}	nd
	SUN-12h	4.33^{b}	-	-	-	8.49^{d}	3.82^{a}	0	1.65^{cd}	1.88^{b}	nd
	SUN-24h	4.31^{b}	8.99^{a}	4.50^{a}	5.74^{b}	9.94^{a}	2.87^{b}	0	1.45^{de}	1.30^{c}	nd
	SUN-M	4.07^{c}	-	-	-	9.72^{b}	2.75^{b}	0	2.73^{a}	0	nd
	SUN-Su	3.43^{e}	7.79^{c}	2.70^{b}	5.51^{c}	8.86^{c}	1.47^{d}	0	1.95^{b}	0	nd
	SUN-Sd	3.35^{f}	7.97^{b}	2.73^{b}	5.89^{a}	8.80^{c}	1.92^{c}	0	1.88^{bc}	0	nd
	SUN-K	3.51^{d}	3.19^{e}	1.70^{d}	2.98^{d}	3.61^{f}	1.60^{d}	0	1.23^{e}	0	nd
Mankessim	MAN-D	6.14^{a}	4.79^{d}	3.64^{d}	2.81^{d}	5.54^{e}	6.72^{a}	3.79^{a}	3.92^{a}	2.99^{b}	nd
	MAN-12h	4.59^{b}	-	-	-	7.63^{d}	5.45^{b}	2.70^{b}	3.81^{a}	1.73^{d}	nd
	MAN-24h	4.35^{d}	8.86^{b}	5.82^{a}	6.65^{b}	9.75^{c}	4.94^{c}	2.50^{b}	2.98^{b}	1.66^{d}	nd

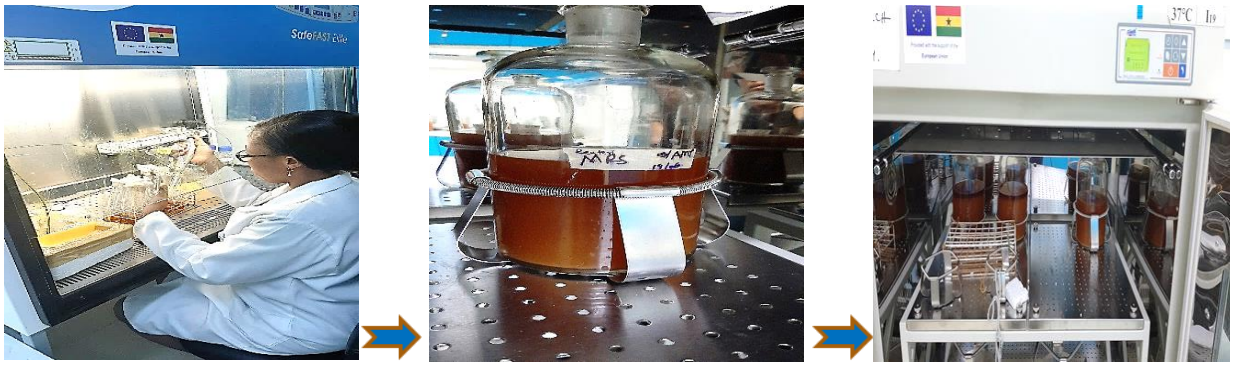
	MAN-M	4.42^{c}	-	-	-	9.99^{a}	4.88^{c}	2.60^{b}	2.50^{c}	3.93^{a}	nd
	MAN-Su	3.43^{e}	8.74^{c}	5.68^{b}	6.54^{c}	9.82^{bc}	3.75^{d}	0.00^{d}	0.00^{d}	1.48^{e}	nd
	MAN-Sd	3.35^{f}	8.94^{a}	5.88^{a}	6.98^{a}	9.98^{ab}	3.55^{d}	1.15^{c}	0.00^{d}	1.95^{c}	nd
	MAN-K	3.95^{d}	3.95^{e}	3.93^{c}	2.57^{e}	4.59^{f}	2.41^{e}	0.00^{d}	0.00^{d}	1.10^{f}	nd
Dodowa	DOD-D	6.27^{a}	3.95^{c}	3.59^{b}	3.88^{d}	5.44^{c}	5.82^{a}	2.71^{b}	2.91^{a}	2.81^{b}	nd
	DOD-12h	4.41^{b}	7.72^{b}	4.87^{a}	5.24^{a}	8.90^{b}	4.54^{c}	1.85^{c}	2.75^{b}	2.59^{c}	nd
	DOD-M	3.98^{c}	-	-	-	8.94^{b}	4.95^{b}	3.91^{a}	2.87^{a}	3.45^{a}	nd
	DOD-Su	3.58^{d}	8.93^{a}	2.76^{d}	4.80^{b}	8.96^{b}	2.67^{e}	2.67^{b}	1.73^{c}	0	nd
	DOD-Sd	3.38^{e}	8.90^{a}	2.91^{c}	4.54^{c}	9.76^{a}	2.94^{d}	1.80^{c}	1.82^{c}	0	nd
	DOD-K	3.56^{d}	2.98^{d}	1.79^{e}	2.10^{e}	3.48^{d}	0	0	0	0	nd
Accra	AMZ-D	6.19^{a}	4.77^{b}	3.45^{d}	2.27^{d}	6.85^{d}	5.44^{a}	2.96^{a}	3.75^{a}	3.76^{a}	nd
	AMZ-12h	4.41^{b}	-	-	-	7.42^{c}	4.81^{b}	2.38^{b}	2.39^{b}	2.62^{b}	nd
	AMZ-24h	4.28^{c}	7.92^{a}	4.98^{c}	5.72^{b}	8.78^{b}	3.34^{d}	1.93^{c}	2.21^{b}	1.47^{c}	nd
	AMZ-M	4.04^{d}	-	-	-	8.94^{b}	3.95^{c}	1.78^{c}	2.36^{b}	0	nd
	AMZ-Su	$3.\ 68^{f}$	7.83^{a}	5.78^{b}	5.65^{b}	8.77^{b}	2.82^{e}	0	1.25^{c}	0	nd
	AMZ-Sd	3.65^{f}	7.86^{a}	5.94^{a}	5.86^{a}	9.94^{a}	2.57^{e}	0	1.15^{c}	0	nd
	AMZ-K	3.79^{e}	3.59^{c}	2.73^{e}	2.68^{c}	4.78^{e}	1.14^{f}	0	1.04^{c}	0	nd

NB: Figures are presented as means and superscript to figures implies significant or not significant at $P \leq 0.05$; nd = Not detected;

- = not enumerated

Appendix 4

Semi-industrial scale starter culture fermentation process



Inoculation of starter in broth Secured in a shaking incubator Incubation of a batch

Ready to be harvested cultures Transferring into centrifuge tubes Centrifugation

Vortexing Some harvested cells Bags of millet at the facility

Destoning of millet Cleaning of millet grain

Weighing of different spices Spices are mixed together and added to millet

Milling in attrition mill Addition of harvested cells to water to be used for kneading

Addition of the water to millet flour Kneading the dough Compacting/compressing

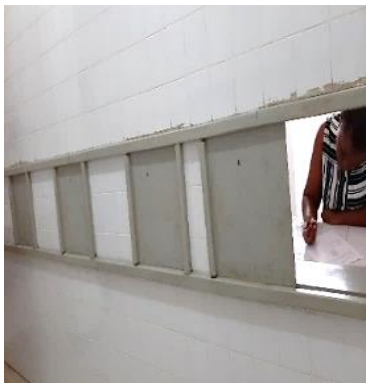
Dough covered to ferment Spreading fermented dough on trays Drying dough in a mechanical dryer

Trays are removed after drying, milled in hammer mill, weighed into pouches, sealed, and pack into boxes

Appendix 5

Sensory (acceptability) evaluation of *Hausa koko*

Prepared porridge



Some panel members accessing the porridge

Some panel members accessing the porridge

Appendix 6

Ballot sheets for sensory (acceptability) evaluation of *Hausa koko*

Name: …… Date: …….....

Tel Number: …… Email: …….....

Instruction:

You have been provided with different samples of fermented millet porridge. Kindly examine each sample and indicate your degree of likeness using the scale below. Please remember to cleanse your palate/mouth with the biscuit and rinse your mouth with the water provided before moving on to the next sample.

Scale/Interpretation

9 = Like Extremely

8 = Like Very Much

7 = Like Moderately

6 = Like Slightly

5 = Neither Like nor Dislike

4 = Dislike Slightly

3 = Dislike Moderately

2 = Dislike Very Much

1 = Dislike Extremely

Attributes	Sample Codes					
Aroma						
Colour						
Consistency						
Taste						
Overall Acceptability						

Which of the product do you prefer most? ……

Why?.....

Thank you for participating

Appendix 7

Publication and conference presentations

Atter, A., Diaz, M., Tano-Debrah, K., Kunadu, A. P. H., Mayer, M. J., Colquhoun, I.J., Nielsen, D.S., Baker, D., Narbad, A. & Amoa-Awua, W. (2021). Microbial Diversity and Metabolite Profile of Fermenting Millet in the Production of Hausa koko, a Ghanaian Fermented Cereal Porridge. *Frontiers in Microbiology*, 1752.

frontiers in Microbiology

ORIGINAL RESEARCH

Microbial Diversity and Metabolite Profile of Fermenting Millet in the Production of *Hausa koko*, a Ghanaian Fermented Cereal Porridge

OPEN ACCESS

Gibson, B., Schwan, R. F., & Zhao, J. (Eds.). (2022). Interspecies Interactions Within Fermented Food Systems and Their Impact on Process Efficiency and Product Quality. Frontiers Media SA. (Citation of Atter et al., 2021 (pg 63-77) in this E-book)

Atter, A*., Diaz, M., Tano-Debrah, K., Kunadu, A. P. H., Mayer, M. J., Nielsen, D.S., Narbad, A. & Amoa-Awua, W. (2021). Bacterial diversity of Hausa koko, a traditional fermented millet porridge in Ghana. Society for Applied Microbiology (SfAM) UK, ECS Research Symposium. 22nd – 26th March 2021.

Atter, A*., Diaz, M., Tano-Debrah, K., Kunadu, A. P. H., Mayer, M. J., Narbad, A. & Amoa-Awua, W. (2021). Potential probiotic *Limosilactobacillus pontis* is the most dominant lactic acid bacteria in Hausa koko fermentation. Research Staff Association Conference, Accra. 19th -21st October 2021.