QUALITY AND MICROBIOLOGICAL STUDY OF BAMBARA GROUNDNUT FORTIFIED INJERA, A FERMENTED FLAT BREAD

By

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Declaration

I declare that the thesis herewith submitted to the Department of Biotechnology and Food Technology, Durban University of Technology, for the award of Masters in Applied Science in Food Science and Technology is my research work and has not been previously submitted for a degree at any other Institution of Higher of Education.

Mellisa Nokulunga Jula Date
Student

As the candidate's supervisor, I agree to the submission of this thesis.

Prof. O.A. Ijabadeniyi Date
Supervisor

Dedication

I dedicate this thesis to my family, especially my mother Thembi Jula and my sister Amanda Jula, and the Jonti Distributors and Gumede family for their unwavering support and love. I would also like to dedicate this thesis to my partner in crime Brian Mngadi, for believing in me and being my anchor in every step of this journey.

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Abstract

Cereal fermented products are popular in developing countries, especially in Asia and Africa, because of their unique taste and fulfilment. Throughout the years, they have played a vital part in bringing up infants as part of their weaning foods and contributing to the daily diet of many households. Food fortification and supplementation of cereal grains with inexpensive readily available legumes, which have higher protein content compared to cereals may lead to a potential decrease in protein-energy malnutrition. Underutilised and indigenous crops such as Bambara groundnut can be in incorporated into the fermentation of cereal fermented foods, such as injera. In this study, injera was prepared by substituting only 9% and 12% Bambara groundnut flour and comparing them with the traditionally fermented original control, which is injera made from only tef flour. The first part of the study was to identify and characterise the lactic acid bacteria (LAB) and yeast involved in the spontaneous fermentation of traditional tef-injera and the newly developed injera fortified with Bambara groundnut (which contains 12% Bambara groundnuts) at different fermentation intervals of 0, 24, 48, and 72 hour. A total of 70 LAB isolates and 30 yeast isolates were identified from both fermentations using rep-PCR fingerprinting followed by sequencing the 16S rRNA gene and the D1/D2 region of the 26S rRNA gene. *Weissella confusa*, *Lc. lactis* and *Lb. curvatus* predominated in both fermentations at different intervals of the fermentation.

The second part of the study investigated the effectiveness of the isolated LAB starter cultures on the production of injera and injera fortified with Bambara groundnut after which their physicochemical properties were evaluated. There was a significant increase ($p<0.05$) in titratable acidity and a significant decrease in pH to below four within 24 hours; recorded for samples inoculated with LAB starter cultures when compared to samples fermented without inoculation. The third and fourth parts of the study investigated the proximate

composition and storage stability of the injera samples. Injera fortified with 12% Bambara groundnut + LAB culture had a significantly high ($p<0.05$) protein of 23.21%, the lowest protein content being Tef injera at 7.35%. The protein digestibility of Tef injera increased with the addition of Bambara groundnut and LAB starter culture. The digestibility of protein increased from 40% for Tef injera to 80% for injera fortified with 12% Bambara flour + LAB culture. There was no significant increase ($p >0.05$) in the amino acid content after the addition of Bambara flour + LAB cultures; the amino acid concentrations were slightly lower than the standard concentration recommended by the Food and Agricultural Organisation/World Health Organisation for adults. Injera samples fortified with Bambara groundnut flour and inoculated with lactic acid starter cultures were stable with microbial counts ranging from 4.42 log cfu/g to 4.68 log cfu/g for TPC at 4 ˚C, yeast and mould, coliforms and aerobic spore formers were not detected in all the samples from day 0 to day three upon storage. Higher counts had been perceived at room temperature ranging from 4.60 log cfu/g to 7.53 log cfu/g for moulds and 4.90 log cfu/g to 9.26 cfu/g for TPC; coliforms were detected in one tef injera only ranging from 4.48 log cfu/g to 6.16 log cfu/g and no detection of aerobic spore formers in all samples. Refrigeration temperatures effectively maintained the microbiological quality of injera for three days. The nutritional quality, distinctively the protein content increased with the addition of Bambara groundnut flour and through the use of lactic acid bacteria as a starter culture

This will potentially pave the way for the commercialisation of injera in the industry with the use of LAB starter culture to ensure a fast and continuous supply of fresh injera that is in high demand.

Keywords: Injera, lactic acid bacteria, Bambara groundnut, rep-PCR fingerprinting

Abbreviations

BF	Bambara flour
FAO	Food and Agricultural Organization
LAB	Lactic acid bacteria
CFU	Coli Forming Units
PDA	Potato Dextrose Agar
MRS	Man Rogosa Sharpe
PCR	Polymerase chain reaction
9% TB	Tef injera fortified with 9% Bambara flour
12% TB	Tef-injera fortified with 12 % Bambara flour
9% TB-LAB	Tef injera fortified with 9% Bambara flour with LAB cultures
12% TB-LAB	Tef injera fortified with 12% Bambara flour with LAB cultures
TF	Tef flour
TIC	Tef injera
TIC-LAB	Tef injera with LAB culture
TTA	Total Titratable Acid
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
RFLP	Restriction Fragment length Polymorphism
PFGE	Pulsed Field Gel Electrophoresis
RAPD	Random Amplification of Polymorphic DNA
AFLP	Amplification Fragment Length Polymorphism
TGGE	Temperature Gradient Gel Electrophoresis
DGGE	Denaturing gradient Gel Electrophoresis

NGS	Next Generation Sequencing
RAPD	Random Amplification of Polymorphic
ND	Not Detected
UPLC	Ultra-Performance liquid Chromatography
HLA-DQ2	Human Leukocyte Antigen
GDP	Gross Domestic Product
USD	United States Dollar
WHO	World Health Organisation

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Preface

This thesis is organised into eight chapters and presented in the format submitted for publication. Chapter 1 is a general introduction to the thesis. Chapter 2 presents a critical review of relevant literature. The areas covered in the literature review include but are not limited to employable strategies to combat protein-energy malnutrition, utilisation of tef seeds, the fermentation and production of tef injera and its nutritional benefits, and methods for microbial population determination in fermented foods. Chapter 3 presents the identification and characterisation of Lactic acid bacteria and yeast involved in the spontaneous fermentation of tef injera and injera fortified with Bambara flour. Chapter 4 presents the physicochemical evaluation of the lactic acid bacteria starter cultures identified and isolated in Chapter 3 for their ability to produce injera. Chapters 5 and 6 investigate nutritional and storage stability of injera after the addition of Bambara flour as a fortificant and the use of LAB starter culture for the fermentation of injera types formulated. Chapters 7 and 8 present the general discussion of the entire findings on the research with conclusions and recommendation for possible future works.

Publications and Conference Outputs

Publications

M.N. Jula and O.A. Ijabadeniyi (2020). Evalaution of Lactic acid bacteria starter cultures for the production of Tef injera and Bambara fortified injera, a fermented flat bread. Italian Journal of Food Science (8012-1 – Under evaluation)

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Conference outputs

Autumn International Scientific Conference on Food Security and Safety- Quality and Microbiological Study of Bamjera (Injera fortified with Bambara groundnuts). 16^{th} -18^{th} May, Johannesburg, South Africa

Chapter 1

Introduction

Injera, a fermented leavened pancake-like sour bread, is the most common staple food in the central, western and northern highlands of Ethiopia, as well as amongst the urban community (Mezimer 2015). Injera is characterised by having eyes or honeycomb-like holes due to the production and escape of carbon dioxide during the fermentation of the injera batter and baking process (Fischer *et al.*, 2014). It is traditionaly prepared from tef flour (*Eragrostis tef*) an ancient cereal, indigenous to Ethiopia (Ashenafi 1994; Fischer *et al.,* 2014). Injera may be prepared from a combination of cereals other than tef, such as barley, sorghum or maize depending on the availability (Ashenafi 1994). Good injera is characterised by its sour taste, softness and ability not to crack when rolled over (Yetneberk *et al.,* 2004). Tef (*Eragrostis tef*) is a highly cultivated crop in Ethiopia, making it the third most produced crop after maize and wheat (Ashagrie and Abate 2012). It is also known to be high in micronutrients such as Fe, Ca, Zinc, B1 vitamin, fibre and is also gluten-free; the very small prolamins make the proteins easily digestible (Bultosa 2007; Abiyu *et al.,* 2013; Girma *et al.*, 2013; Mezemir 2015). Tef-injera traditionally is fermented for two to three days by mixing the tef flour and water, and in some cases, a previous successful batch is used as the inoculum (Abiyu *et al.,* 2013; Fischer *et al.,* 2014; Mezemir 2015). Indigenously fermented foods, such as injera have been part of people's diets for centuries providing nutrients and a manner of preserving and cooking foods that have contributed to food security especially in developing countries of the world (Senapati *et al.,* 2016).

Food insecurity and nutritionally related diseases like protein-energy malnutrition are still a major problem in developing countries. Traditional fermented foods such as mahewu, ogi, and injera are staple foods, providing meals for millions of people, but they are low in essential amino acids and

proteins. The market price of tef has increased tremendously compared to other cereals due to the high demand versus the low production. (Agza *et al.,* 2018). Co-fermentation with under-utilised legumes that are a rich source of protein could be a way of fighting protein-energy malnutrition as meat is an expensive source of protein and most people in the developing world cannot afford animal protein (Ghebrehiwot *et al.,* 2016). Bambara groundnuts (*Vigna subterranea*) is a good source of macro and micronutrients there is a need to promote the production, processing, and consumption of the legume to combat malnutrition (Muhammad 2014; Mabhaudhi *et al.,* 2016). Bambara groundnut is a highly nutritious with protein ranging between 18% to 27% (Abdualrahman *et al.,* 2012; Arise 2016), carbohydrate (65%), fat (6.5%), phosphorus (380 mg/100g), calcium (73 mg/100g) and methionine (1.8-2.84%) (Stephens 1994; Ajibade 2018). The practice of combining cereal flours with legume flours to improve the nutritional and quality of fermented products is not new a new practice. It has proven to be successful in elevating the nutrients of a food, and it continues to be practised. Incorporating Bambara groundnut or legumes to fermented products such as ben-saalga (Tou *et al.,* 2007), kissra (Abdualrahman *et al.,* 2019) and Ogi (Ajanaku *et al.,* 2012) has been proven to substantially increase the protein, ash and lipid content of the fermented products.

The safety and quality of these spontaneously fermented foods is another major concern, as indigenous processing knowledge is not well documented, leading to processes being conducted on a trial and error basis jeopardising the quality and safety of the food. Other factors include labour extensive techniques, long fermentation times, low productivity and fermentation failures (Pandey *et al.,* 2016; Senapati *et al.,* 2016). Previous work on African fermented foods has revealed a complex and significant microbial diversity responsible for inherent desirable characteristics. *Lactobacillus, Lueconostoc, Pediococcus and Weissella* species are the most predominant LAB

species (Adimpong *et al.,* 2013). The introduction of a starter culture can prove to be beneficial in improving the product quality through rapid accelerated metabolic activities and improvement of the fermentation process resulting in desirable sensory attribute and improved safety (Freire *et al.,* 2015). A rapid pH drop from 6.0 to 4.0 and capacity to liberate amino acids were observed for these products after inoculating with single and mixed lactic acid bacteria starter cultures; kunun-zaki (Onyimba *et al.,* 2017), bushera (Muyanja *et al.,* 2012), togwa (Mugula *et al.,* 2003) and enturire (Hassen *et al.,* 2018). Microorganisms associated with injera fermentation have previously been identified as lactic acid bacteria, yeast and some enterococci species. Fischer *et al.* (2014) employed culture-independent methods to identify phytic acid degrading LAB possibly responsible for injera fermentation. Molecular-based techniques allow for a 90-99% detection level of microorganisms within a community without discriminating between the living and the dead (Meroth *et al.,* 2003; Nielsen *et al.,* 2007; Cocolin *et al.,* 2013). The unambiguous identification of LAB and yeast from injera using molecular biology-based methods is the first step in the future development of defined starter cultures as a feasible means of optimising the fermentation of tef-injera and injera fortified with Bambara groundnut.

This present study is designed to understand the fermentation process of injera and better optimise the fermentation process of injera and improve its nutritional composition by fortifying with Bambara groundnut flour.

1.1 Problem statement

A large proportion of plant-derived foods consumed worldwide are cereals which are processed by fermentation; therefore, it is important to reduce the anti-nutritional factors in order to increase mineral bioavailability, protein and starch digestibility (Moroni *et al.*, 2015). The implementation and identification of starter cultures for small scale fermentations are important to improve the fermentation process and the quality of fermented foods. The use of LAB starter cultures can improve the safety of fermented foods, such as injera and also reduce the fermentation time without compromising the quality of the finished product (Sanni *et al.*, 2002; Ogunremi *et al.*, 2017).

Legumes are valuable and nutritious because of the vitamins, proteins and minerals but are not utilised hence underutilised. Food fortification and supplementation with legumes such as Bambara groundnut are an inexpensive and practical means of increasing the amino acid and protein content of food (Ajanaku *et al.*, 2012; Pasqualone 2018).

To date, there is no reported work on the fortification of injera with Bambara groundnut, including its controlled fermentation. Furthermore, there is little or no work, especially in South Africa on the use of molecular-based techniques to study the microbial community of injera and injera fortified with Bambara groundnut. This research study will also bring about the awareness of Bambara groundnut as an inexpensive fortifying agent that has the potential to gradually improve the protein quality and overall nutritional content of foods, such as injera that are staple foods with poor protein quality.

1.2 Aim

To optimise injera production using defined LAB starter cultures and improve the protein content by fortifying with Bambara groundnut.

1.3 Hypotheses

- Molecular-based techniques such as rep-PCR coupled with the sequencing of the 16S rRNA and 26S rRNA gene allow for a 90-99% detection level; therefore, the use of these molecular techniques will result in successful identification and characterisation of LAB and yeast species involved in the spontaneous fermentation of tef-injera and injera that is fortified with Bambara groundnut (Cocolin *et al.,* 2013; De Filippis *et al.,* 2017).
- Incorporating Bambara groundnut into the injera fermentation will lead to a finished product with improved protein content, and overall nutritional content. Bambara groundnut is a rich source of protein (18-24%) with a good source of amino acid with relatively higher proportions of lysine and methionine when compared to other legumes (Eltayeb *et al.,* 2011).
- Fermenting injera with starter cultures will result in rapid acid production within 24 hours; leading to a pH< 4.0 and possibly improve the nutritional quality of injera inoculated with LAB starter culture. The introduction of starter cultures for small scale fermentations has been proven to improve processing conditions, improve product quality and nutrition (Freire *et al.,* 2015).
- Spontaneously fermented injera and the Bambara fortified injera will be contaminated with undesirable microflora such as wild yeast, mould and other pathogenic bacteria naturally

found in the flour when compared to injera inoculated with LAB starter cultures (Waters *et al.*, 2015). Ogi fermented with LAB cultures were found to inhibit gram negative pathogens such as enterotoxigenic *E.coli*, *Campylobacter* as well as gram positive bacteria such as *S. aureus* (Nout 1994).

- Storing tef-injera and the Bambara fortified injera samples at a lower temperature of 4˚C will result in a more stable injera than when kept at higher temperatures such as 10˚C and 25˚C.

1.4 Objectives

1. To identify and characterise lactic acid bacteria and yeast using rep-PCR fingerprinting and sequencing the 16S rRNA and 26S rRNA gene.
2. To produce injera fortified with Bambara groundnut using LAB starter cultures.
3. To determine the approximate composition, amino acid and protein digestibility of tef flour, Bambara flour, injera and injera fortified with Bambara groundnut (both controlled and spontaneously fermented).
4. To determine the microbial activity during the fermentation of injera and injera fortified with Bambara groundnut (both controlled and spontaneously fermented).
5. To determine the storage stability of injera and injera fortified with Bambara groundnut (both controlled and spontaneously fermented) stored at 25˚C and 4˚C.

Chapter 2

Literature Review

2.1 PROTEIN-ENERGY MALNUTRITION

The number of undernourished people in the world rises every year, and in 2016, the estimated number of undernourished people increased from 777 million in 2015 to 815 million (FAO. *et al.*, 2015). Climate conditions such as El Nino/La Nino related phenomena have affected most regions worldwide, but the most noticeable regions are those in Africa and Asia. Poverty remains a major challenge in many countries in Sub-Saharan Africa and other developing countries. According to (Hazell and Haddad 2001), about 1.2 billion rural people live in poverty (defined as living on less than $1 per day) (Hazell and Haddad 2001). About nine percent of these people live either in Asia or Sub-Saharan Africa (Ijabadeniyi and Jula 2015). The inability of households and individuals to access food of adequate quantity and quality (containing proteins, carbohydrates, vitamins) is an important determinant of malnutrition (McGuire 2015; Daryanto *et al.*, 2016). Malnutrition is not necessarily the result of a lack of access to sufficient, nutritious and safe food. Other food factors such as sanitation, hygiene, drinking water, education and healthcare also attribute to malnutrition (FAO 1985; McGuire 2015). Tubers, roots and cereals are major staple foods forming part of the main diet in African countries and are nutritionally inadequate, failing to meet many micro and macronutrients needs (Ernest *et al.*, 2013; Suri *et al.*, 2014). The heavy consumption of cereal or starchy foods which are low in protein and some micronutrients in African and Asian countries has resulted in protein-energy malnutrition (PEM), a type of malnutrition, defined as a variety of pathological conditions arising from a lack of adequate protein and calorie intake (Ernest *et al.*, 2013). PEM is characterised by poor weight gain, slowing of linear growth, behavioural changes

and attention deficits, threatening children aged between 1 and 10 years in developing countries (Temba *et al.,* 2016; Ajibade 2018). PEM has been identified as the cause of kwashiorkor, marasmus and marasmic-kwashiorkor, which are associated with other daily diseases such as malaria, diarrhoea, anaemia and tuberculosis. The introduction of available, inexpensive protein food sources such as legumes is a feasible means of conquering malnutrition, in particular, PEM. Legumes may contribute up to 80% of dietary protein (Adebayo and Balogun 2018).

2.2 CEREAL AND CEREAL FERMENTED FOODS

Cereals apply to many grain genera that are flowering plants all belonging to the grass family (*Poaceae)*, whose seeds are used as foods. The major cereal grains of economic importance are the cool season crops, including wheat, barley and oats; and the warm season cereals including rice, maize, sorghum and the millet (Wrigley *et al.*, 2015). Cereals, as a source of calories, are the basic food of man since prehistoric times and the global production of cereals was about 3 billion metric tons in 2014.

Cereals are major crops not only in Africa but worldwide, and they are grown in over 73% of the total world harvested area contributing to over 60% of the world's food production with the annual world production of all cereals grains now approximately 3 billion tons (Charalampopoulos *et al.,* 2002; FAO 2013; Galati *et al.,* 2014). Cereal grains have an adequate amount of sulphur-containing amino acids but are deficient in lysine, methionine, isoleucine and tryptophan (Achi and Ukwuru 2015; Wrigley *et al.,* 2015). Cereals are good fermentable substrates, leading to a variety of fermented foods. Fermented cereal-based products are mainly derived from the major cereals and can be differentiated according to their texture. Fermented cereal foods are either liquid (porridge) or stiff gels (solid). Cereal porridge includes but is not limited mahewu, mawe and ogi

and cereal gels include but is not limited to agidi, kenkey, bogabe, banku, injera and kisra (Taiwo 2009; Achi and Ukwuru 2015). The high levels of carbohydrates, vitamins and dietary energy, insoluble dietary fibre, minerals, phytochemicals and other antioxidant activities, supplementation and fortification of cereals with low-cost crops like legumes may improve the nutritional contents (Noorfarahzilah *et al.*, 2014). A wide range of fermented products is produced using cereals at household to semi-industrial and industrial scales. However, during their production, nutrients such as proteins and minerals, are lost from the cereal grains; thereby affecting the nutritional quality (Ogunremi *et al.*, 2017).

2.3 EMPLOYABLE STRATEGIES TO COMBAT PROTEIN-ENERGY MALNUTRITION

2.3.1 FOOD FORTIFICATION AND SUPPLEMENTATION

Food fortification is a method of adding bioactive nutrients to edible products or non-nutrient elements. The objectives of fortification are to correct or prevent nutrient intake shortcomings in nutrient consumption and related deficiencies, to balance the total nutrient profile of a diet, to restore nutrients lost in processing or to appeal to consumers seeking to complement their diet (Dwyer *et al.*, 2015). Foods suitable for fortification must be staple foods consumed adequately in that population (Oyeyinka and Oyeyinka 2018). Types of commercial fortification programs that have been recruited in different parts of the world to cater for different populations and deficiency include mass fortification, targeted fortification, voluntary fortification and mandatory fortification (Miller and Welch 2013). Fortification was first introduced in the United States in 1924, when iodine was voluntarily added to salt to reduce the incident of endemic goitre, decreasing the incident from 35% to 2.5% between the period of 1924 to 1935 (Dwyer *et al.*, 2015).

Fortification of plant-based complementary foods can be an effective strategy for addressing malnutrition. Also, fortification of cereal-based foods with protein-rich legumes has been identified as a possible means of alleviating PEM among low-income countries.

Bambara groundnut over time has shown its potential for the fortification of traditional weaning foods in Africa. Boiled Bambara groundnuts were added to fermented maize dough, increasing the protein content from 10.0% to 16.4% and an increase in moisture content, fat, ash, lysine and tryptophan compared to unfortified maize dough (Mbata *et al.,* 2009). Cooked banana in Nigeria as a weaning food have been enhanced through supplementing with fermented Bambara groundnut flour, and the formulated food contained 70% cooked banana and 30% Bambara groundnut flour (Mkandawire 2007).

2.3.2 CO-FERMENTATION TO IMPROVE THE NUTRITIONAL CONTENT

Most fermentations or fermented products of African and Asian descent are mainly of cereal crops. Cereals do not offer the best quality of micro- and macronutrients; as a result, the nutritional quality and sensory properties of the resulting product are typically poor in collation with dairy or milk products due to their low protein content, starch, and essential amino acid availability (Nout 2009). The problem also lies with the presence of anti-nutrients found naturally in the plant source such as phytic acid, tannins, and polyphenols, which prevent the absorption of bioavailable micronutrients by the body (Blandino *et al.,* 2003). Spontaneously fermenting cereal foods can have detrimental effects on the carbohydrate levels resulting in low levels, as well as some non-digestible poly and oligosaccharides. The availability for enzymatic degradation of phytate may be provided by optimum pH due to fermentation; the availability and a possible increase in the amount of soluble zinc, magnesium, calcium and iron, and proteins attributing to the decrease in

phytate content or concentration (Nout 2009; Baye *et al.*, 2013). Phytate is present in legumes or grains as a complex with povilant cations such as proteins and iron. (Blandino *et al.*, 2003). Co-fermentation of cereal crop with legumes has proven to be an effective method of improving the quality of cereal fermented products without breaking the pocket (Granito and Alvarez 2006). Legumes are mostly grown and cultivated in developing countries and have exceptional nutritional content. The decrease in consumption, as well as cultivation, is mainly due to a hard to cook phenomenal and intestinal discomfort when consumed. Fermenting of legumes has proven to decrease these problems and increase the nutritional quality of cereal food fermented foods. A study conducted by (Egounlety 2002) resulted in the production of high protein-energy food with a good nutritive value by co-fermenting Ogi, a maize fermented porridge consumed by infants with different legume crops such as soya, cowpea and Bambara groundnuts. Protein and energy values were in between 15.55 and 19.30 % and 17505 and 18726 KJ/Kg, respectively. Ben-saalga a traditionally fermented millet-based gruel was also co-fermented with Bambara groundnut an African legume to improve its nutritional quality giving rise to a product with appropriate macronutrients and high energy at a suitable consistency compared to the original ben-saalga (Tou *et al.*, 2007). Sorghum Ogi fortified with Bambara groundnut in an effort to increase its nutritional value, the protein, amino acid content of the fortified Ogi increased significantly as a result (Opere *et al.*, 2012).

2.3.3 LACTIC ACID BACTERIA AS A FUNCTIONAL STARTER CULTURE

Controlled fermentations, whereby starter cultures are used to ferment foods are more desirable because the starter cultures isolated, characterised and maintained for use as fermentative microorganisms, are directly added to the substrate or raw material in large quantities and fermented at optimal conditions (Hansen 2002; Bourdichon *et al.*, 2012). Commonly fermented

food products such as sauerkraut and yoghurt are primary fermented using lactic acid bacteria starter cultures, resulting in flavour and aroma production, proteolytic, lipolytic activities, inhibition of undesirable microorganisms and providing particular characteristics through the fermentation in a more controlled and predictable manner (Holzapfel 2002; Holzapfel and Schillinger 2003; Casaburi *et al.,* 2016).

Commercially there are two groups of available lactic acid bacteria starter cultures, which include defined starter cultures consisting of single or multiple known strains usually isolated from a certain food due to their fermenting action and secondly, undefined starter culture consisting of unknown mixed strains (Holzapfel and Schillinger 2003). Not all strains of species are suitable for use as starter cultures. The modern selection of strain involves a single or multiple strains selection specifically for the adaptation to a substrate or raw material. Normally the fermenting strains are selected at strain level (Giraffa 2004). Spontaneously fermented foods present a diversity of metabolic activities which may differ among strains of which pure cultures are isolated from, these include differences in development rates, adaptation to the substrate's ability to breakdown anti-nutrient factors antimicrobial properties, flavours and quality attributes and competitive growth behaviour in mixed cultures. The selected microorganisms used in commercial fermentations are those identified from spontaneous fermentations (Mehta *et al.,* 2012).

The use of lactic acid bacteria to ferment foods can contribute strongly to the flavour composition of foods and beverages. Flavour and aroma are vital characteristics in sensory properties and acceptability of the fermented foods. Changes depend largely on the starter culture used, the cereal substrate being fermented and other processing methods (Brandt 2014; Nionelli *et al.,* 2018). Flavours are produced due to the lactic acid utilising the sugar resulting in lowered acidity, which produces organic acids. The lowered pH also affects the endogenous cereal enzymes involved in

the flavour compound generation or other precursors. Also, lactic acid bacteria produce amino acids and other functional flavour associated metabolites (Hammes *et al.,* 2005).

Starter cultures do not necessarily guarantee a successful and safe fermentation when they are used to initiate the fermentation process, whether it is small or large. It is, therefore, important to consider other contributing factors for a safe and successful fermentation, with or without inoculation of microorganisms. Safety measures such as good manufacturing practice and good hygiene practices should be employed. The condition of the grains used, water containers and machinery will affect the fermentation (Steinkraus 1997).

2.4 CULTURE-DEPENDENT AND CULTURE-INDEPENDENT TECHNIQUES FOR IDENTIFYING MICROORGANISMS

Microorganism have been identified and characterised based on their phenotypic properties which include their morphology, method of glucose maturation, growth at different temperatures, lactic acid configuration, the fermentation of various carbohydrate, the methyl esters of fatty acids and the pattern of proteins in the cell wall or the whole cell (Mohania *et al.,* 2008). These methods have restrictions such as poor reproductivity, ambiguity in identities due to poor discriminatory powers and the inability to characterise minor populations of microorganisms which require selective enrichment, inadequacy of detection of non-cells, time consumption and labour demands (van Hijum *et al.,* 2013; Tamang *et al.,* 2016). Accurate identification of microbial species in food samples is important for quality, ecology and the safety of food. Conventional culture-based methods termed as culture-dependent techniques have been used for centuries to identify microorganisms in the environment, such as in foods, soil, water (Madoroba 2009). Culture-dependent techniques rely on the isolation and cultivation of microorganisms before their identification and typing of which have not been entirely successful throughout for complete

microbial characterisation of numerous ecosystems; and stressed or damaged cells are not easily identified (Cocolin *et al.,* 2011; Mayo *et al.,* 2014a). Some microorganisms grow in natural habitats which cannot be reproducible by laboratory media. The largest drawback of culture-dependent techniques is the inability to describe the biodiversity in complex ecosystems correctly (Cocolin *et al.,* 2011). *Cyanobacterium sp*. could not be identified in cold-smoked salmon due to inhibition of its growth by sodium acetate in de-Mann, Sharpe and Rogosa (MRS) medium (Madoroba 2009). Gonzalez *et al.,* (2000) identified 249 LAB isolates from freshwater fish and their environment using 44 morphological and physiological with a high identification percentage of 90% at genus level which demonstrated low taxonomic resolution for a labour-intensive technique.

The invention and introduction of a polymerase chain reaction (PCR) have led to new strategies for studying microorganisms, making it possible to investigate microorganisms without cultivation. Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA) are substrates utilised for PCR amplification, and the target genetic materials from the desired microorganisms through the use of primers which amplify the target sequence within the given population (Justé *et al.,* 2008; van Hijum *et al.,* 2013; Ruiz *et al.,* 2014; Zhao *et al.,* 2015). PCR primers are needed in order to detect different LAB taxa or strains and can be designed for amplification at any taxonomic level, universal primers targeting the V3 or V6-V8 region of the 16S rRNA genes allows for the bacterial community to be analysed. In the case of an ecosystem with a relatively diverse bacterial flora, combining both the V3, V6-V8 amplicon-type can result in clear and successful identification of bacterial species (Temmerman *et al.,* 2004). Kaufmann *et al.,* (1997) designed a primer that amplified a 16S rDNA fragment specific for bifidobacterial species in a food matrix which allowed for the detection of bifidobacterial isolates at the genus level.

Bacterial ribosomal regions such as 16S, 23S rRNA and the D1/D2 region of the 26S rRNA of the yeast are ubiquitously conserved gene regions targeted by PCR primers, and this is because of their universal abundance. PCR based methods such as rep-PCR fingerprinting and restriction fragment length polymorphism (RFLP), pulsed field gel electrophoresis (PFGE), Random Amplification of Polymorphic DNA (RAPD), and Amplification Fragment Length Polymorphism (AFLP) are regarded as important for the specific characterisation and detection of LAB strains (Figure 1). Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) analysis of the faecal 16S rDNA gene and its rRNA amplicons have shown to be powerful approaches in the determination and monitoring of bacterial community in faeces (Olive and Bean 1999; Mohania *et al.,* 2008; Wang and Salazar 2016).

Rep-PCR was introduced to differentiate microorganisms by combining the advantage of DNA amplification with the application of repetitive sequence-based oligonucleotide primers. Prokaryotic and Eukaryotic genomes contain dispersed repetitive sequence separating longer single copy DNA sequence. Genomic fingerprinting with interspersed repetitive sequence-based probes distinguished unrelated organisms because individual bacterial strains vary depending on the distance between the repetitive sequence (De Vuyst *et al.,* 2008; Tafvizi and Tajabadi Ebrahimi 2015). Rep-PCR based on outwardly facing oligonucleotide primers complementary to interspersed repetitive sequences will enable the amplification of differently sized DNA fragments consisting of sequences lying between the elements. Rep-PCR specific primers which amplify repetitive bacterial DNA elements include ERIC, BOX or $(GTG)_5$, with primer $(GTG)_5$ has proven to be effective the differentiation of Lactobacilli and bifidobacteria at the species level and potentially on strain level. Multiple amplicons of different sizes can be fractionated by

electrophoresis and enable the establishment of the DNA fingerprinting patterns specific for individual bacterial clones (Versalovic *et al.,* 1994). Environmental samples, such as food samples, are subjected to DNA extraction from the bacterial species occurring in the sample. The extracted DNA mixture is then used as template in PCR amplification of variable DNA regions of taxonomic interest by obtaining an amplified product that is a mixture of amplicons from the species present in the initial sample and then separated by DGGE, which entails running the amplicon mixture directly on agarose gels to obtain the fingerprint bands (Figures 2 & 3). The bands from the gel may be directly cloned and sequenced to allow the identification of individual communities. Sequences obtained are then processed by trimming and filtering and then compared with a public database, GenBank to obtain the identity and characteristics of microorganism (Benson *et al.*, 2004; Adimpong *et al.,* 2012). These molecular-based techniques have now been termed as culture-independent methods, offering numerous advantages over culture-based methods.

Next-generation sequencing (NGS) is a collective term describing several technologies that achieve massively parallel sequencing of heterogenous DNA fragments. Tools such as phylobiomics, metagenomics and metatranscriptomics use parallel pyrosequencing of tagged 16S rRNA gene amplicon providing detailed information on microbial communities, required for sensory quality, safety improvements and starter culture design for commercial use (Tanigawa and Watanabe 2011; Nguyen *et al.,* 2013; Mayo *et al.,* 2014b; Stefanovic *et al.,* 2017). The benefits of NGS over first-generation profiling methods such as DGGE and TTPFL is the capability of NGS techniques to compare communities by phylogenetic similarity and by extension functionality in addition to count-based metrics. This can be impactful in observed microbial succession, demonstrating the impact of conditions or treatments not only on microbial diversity counts but also functional processes. Limitations or issues with using NGS include computational analysis,

advanced bioinformatics algorithms, power and storage to handle complex data produced. These techniques have some challenges but can be useful if carefully selected in order (Bokulich and Mills 2012).

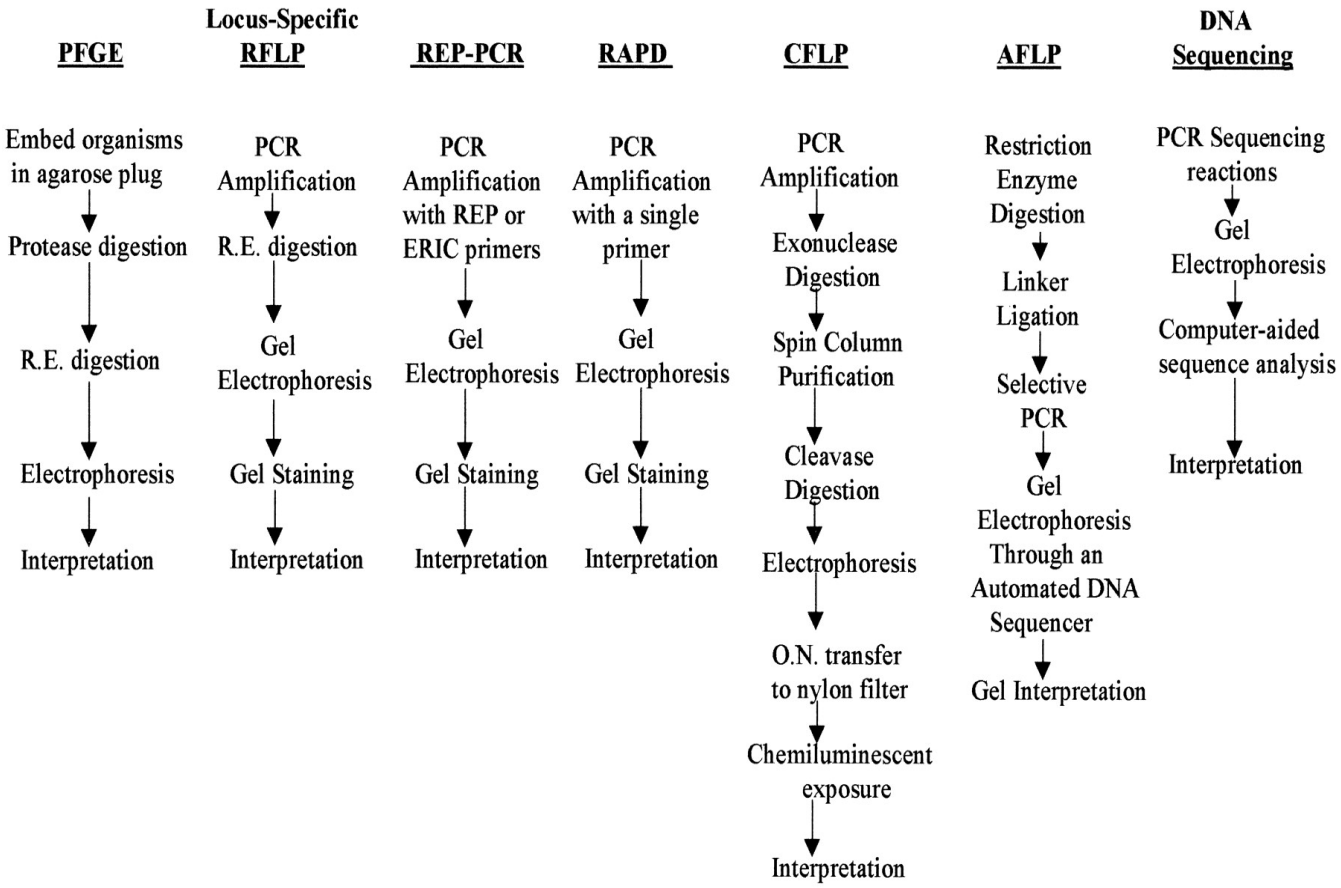

Figure 1: Main steps involved in DNA-based methods for characterisation of LAB. (R.E. represents restriction enzyme) (Adopted and modified from: (Olive and Bean 1999).

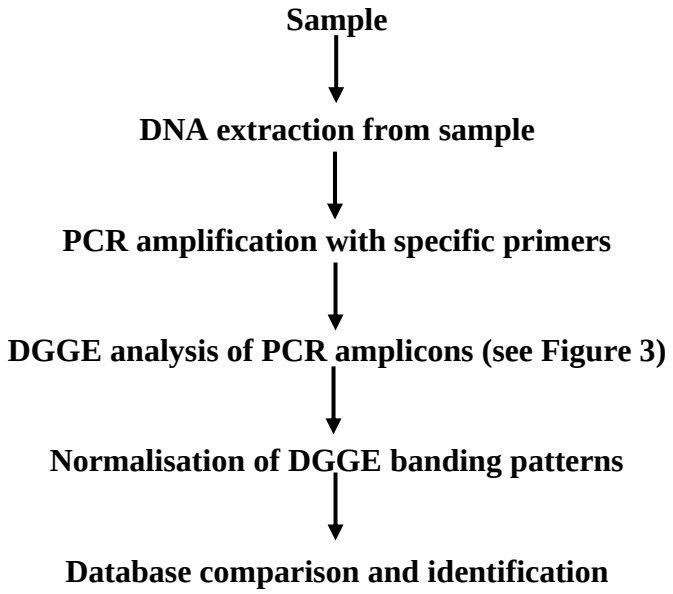

Figure 2: Schematic overview of the DGGE method for the microbiological analysis of unkown sample (Temmerman *et al.*, 2004).

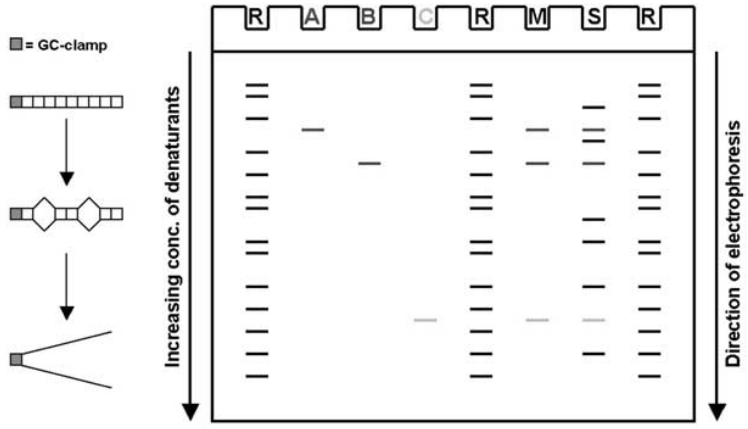

Figure 3: PCR amplicons of equal lenghts are electrophoretically separated in a sequence dependent manner, separating the double stranded DNA amplicons to single stranded DNA through melting domains (Temmerman *et al.*, 2004).

2.5 ERAGROSTIS TEF

Tef (*Eragrostis tef* (Zucc.) Trotter) belongs to the family of Poaceae subfamily Eragrostoideae and the genus Eragrostis. The genus is said to generally contain an estimated number of 300 species (Ketema 1997). The common name of Eragrostis tef (Zucc) Trotter is tef/teff which is derived from the Ethio-Semitic root "tff" which means lost, a reference to its small seed size, weighing 0.264 kg (Figure 4) (Ketema 1997; Kaleab 2014). Tef is a plant that cycles carbon dioxide into four carbon sugar compounds to enter the Calvin cycle. It is a self-pollinated and chasmogamous annual cereal. Tef has a fibrous root system with mostly erect stems, similar in appearance to bunch grasses with some cultivars being the bending or elbowing type (Ketema 1997).

The sheaths of tef are smooth, globous, open and shorter than internodes. The ligules are very short and ciliated while its lamina is slender, narrow and nearly linear with elongated acute tips. Tef has an inflorescence panicle showing different forms, from loose to compact; the latter looks spiky (Figure 5). The spikelets of the tef grain have 2-12 florets with each floret comprising of a lemma, palea, three stamens and ovary and mostly two or three feathery stigmas. The caryopsis of the seed is 0.9-1.7mm in length and 0.7 -1.0m, in diameter. Bultosa (2007) tested the weights of 12 different tef varieties and obtained an average of 0.246g. The colour of tef seeds is diverse as it varies from white (ivory) to dark brown (black) as depicted in Figure 6. In Ethiopia, tef grains are divided into three major classes identified in Figure 6 as white tef (nech), red tef (quey) and mixed tef (sergegna) (Kaleab 2014). The thickness of the testa located within the pericarp varies in colour with red tef having a thicker testa compared to the white, which is filled with pigment materials (Abebe *et al.*, 2015).

Tef (*Eragrostis tef* (Zucc)Trotter) is considered one of the smallest grains in the world but is also one of the most important cereal crops in Ethiopia (Belay *et al.*, 2007). This endogenous Ethiopian crop is highly utilised, with the largest share of the area (23.42%, 26 million hectares being used

to cultivate this crop (Mengesha 1966; Bultosa 2007; Mezemir 2015). The consumption of tef seeds was first recorded in Ethiopia; the only country that cultivates tef seeds for human consumption. In other countries such as Australia, the United States of America, South Africa and Kenya, tef is cultivated for animal consumption (Mengesha 1966). Tef is second to barley in grain yield, contributing up to two-thirds of the protein in-take of the Ethiopian population (B. M. G. Jones *et al.,* 1978). Tef seeds are extremely small, and difficult to decorticate; therefore, it is consumed as a whole grain (Kaleab 2014)

Tef is said to have been introduced to Ethiopia before the Semitic invasion of 1000 to 4000 BC, according to (B. M. G. Jones *et al.,* 1978; Belay *et al.,* 2005). The cultivation of this crop occurred before the ancient introduction of emmer and barley (Belay *et al.,* 2005).

Tef (*Eragrostis tef* (Zucc)Trotter) seems to have originated in Africa with 43% of the genus *Eragrostis,* 18 % in South America, 12% in Asia, 10% in Australia and 9% in North America and 2% in Europe. 14 % of the 54 listed are endemic to Ethiopia (Costanza *et al.,* 1979). The genetic diversity of tef exists nowhere in the world except in Ethiopia, an indication that it originated and was domesticated in Ethiopia (Ketema 1997). An investigation by Jones *et al.* (1978) on the domestication and origin, revealed species related to tef. The difference noted between tef and related species was the absence of glands. Progenitors of tef are, therefore, likely to have glandular plants, and the representative of closely related species may provide evidence of the origin of the area of domestication. B. M. G. Jones *et al.* (1978) did a survey of the collection of *E. pilosa, E aethiopica, E, cilianensis, E. minor* and *E. barielier* at the herbarium of the Royal Botanic gardens, to ascertain the distribution of eglandular plants of these taxa in Africa, Asia, and Europe. She concluded that combining geographical data obtained for all the species examined, they may be a

concentration of englandular forms in Africa, notably in the North East (Ethiopia, Sudan, Egypt, Uganda, and Kenya).

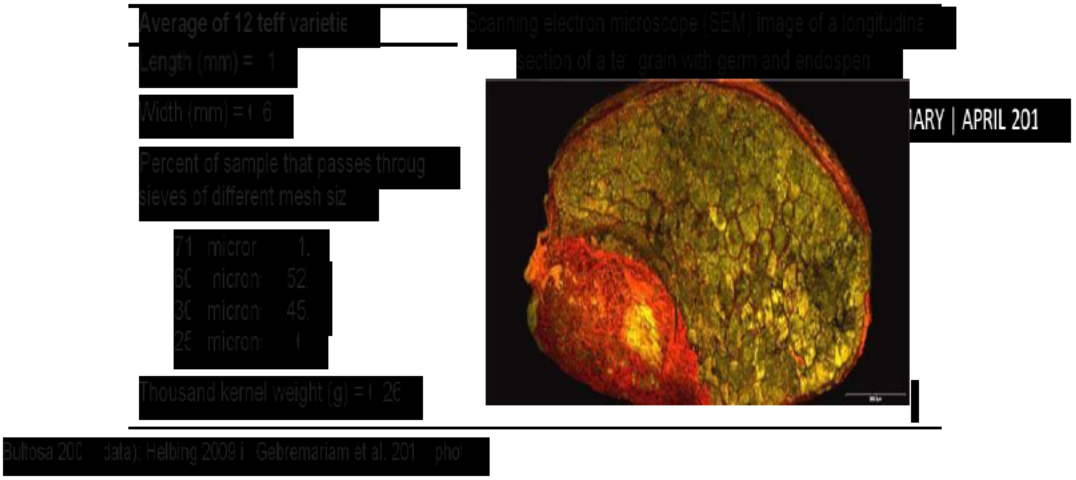

Figure 4: Scanned electronic illustration of tef grain with germ and endosperm, the longitudinal section (Kaleab 2014).

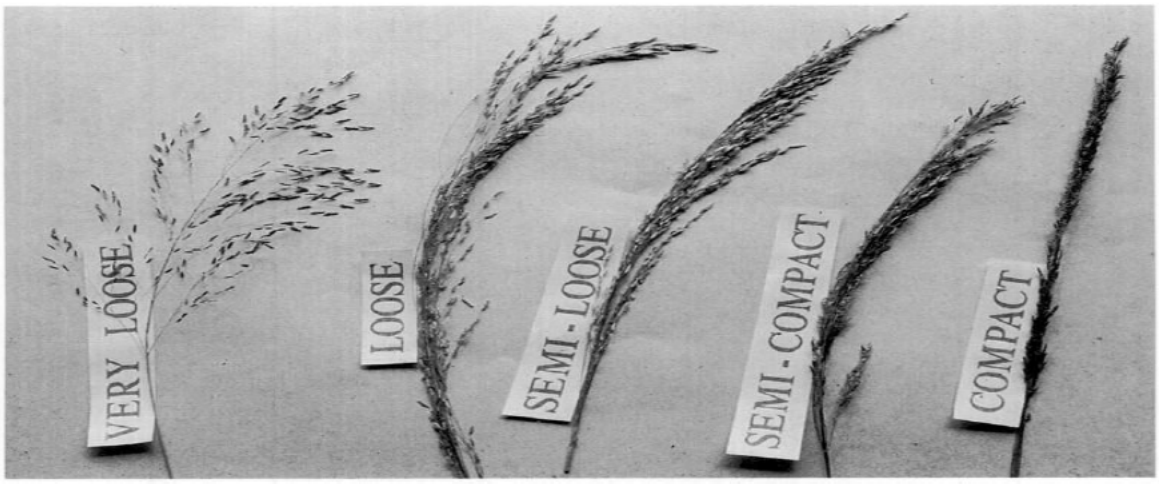

Figure 5: Types of panicles (very loose, loose, semi-loose , semi-compact, compact (Kaleab 2014))

Figure 6: Tef seed varieties from Ethiopia; white, brown and red tef (Uliasz 2015)

2.5.1 NUTRITIONAL COMPOSITION OF TEF SEEDS

The protein content of *Eragrostis Tef* is similar to that of millet. A large portion of the small cereal is the embryo, rich in protein (Abebe *et al.,* 2015). Tef has an average protein content ranging between 8-11%, glutelin and albumin being the major protein storage components, and their order of fractional importance being, glutelin (44.55%) > albumins (36.6%) > prolamins (11.8 %) > globulins. Proteins play numerous vital roles, and some important roles include determining the texture of a food and thickening, binding or as a gelling agent and emulsifying or foaming agent recast (Kaleab 2014; Abebe *et al.,* 2015).

The distribution of these proteins varies among different cereals, and the solubility also varies. Albumins range from 4% in maize to 44 to rye, globulins from 3% in maize to 55% in oats, prolamins from 2% in rice 55% in maize and glutelin from 23% in oats to 78% in rice. Among these protein fractions in cereals, prolamins fraction is the most studied. Prolamins are

characterised by a particular amino acid composition of domains with a high content of proline and glutamine. It has been demonstrated before that these protein domains are resistant to degradation by gastric acid, pancreatic and proteases in the human gut (Ketema 1997; Gebremariam *et al.,* 2014a; Kaleab 2014).

Tef has been recorded to have an excellently balanced amino acid composition which makes it comparable to the amino acid composition of an egg, although it has lower lysine content (Kaleab 2014). The lysine content of tef is higher when compared to other cereals such as wheat and sorghum (Table 1). Isoleucine, leucine, valine, tyrosine, threonine, methionine, phenylalanine, arginine and histidine contents of tef are higher than in barley, wheat, and most other cereals. Isoleucine, valine, tyrosine, serine and glycine contents of tef are slightly lower than those of brown rice (Gebremariam *et al.,* 2014b).

2.5.1.1 Carbohydrates

Tef grains are h composed of complex carbohydrates, making up 80% of the tef grain. Tef is a starchy cereal with a 73% starch content (Kaleab 2014) which is higher than most other cereals (Table 1), making it a potential gluten-free cereal that can replace wheat and other cereals in their application as a source of food energy. Tef starch granules are very small (2- 6µm in diameter) and similar in size to rice granules (2-10 µm) but larger than Amaranthus (1-2µm) and quinoa (0.5-3µm) starch granules(Bultosa and Taylorb 2004). The shape is polygonal, smooth with no surface pores. The composition of tef starch granules is similar to other normal native cereal starches with 25- 32 % amylose (Ketema 1997; Gebremariam *et al.,* 2014a; Kaleab 2014).

2.5.1.2 Fats

Cereals are naturally a good source of essential fatty acids, which are required by the human body for excellent growth development and long-term health. Bultosa (2007) found that that the crude

fat content of tef flour ranged between 3.0-2.0 % with a mean of 2.3 %. The crude fat of tef is generally higher than that of wheat, rye, and brown rice but lower than that of barley, maize, sorghum and pearl millet. Tef grains are rich in unsaturated fatty acids. The seed contains 22 w/w of fixed oil-rich unsaturated fatty acids (72.46%) among which oleic acid is predominant (32.4%) followed by linoleic acid (23.83). Tef seeds are significantly small, which makes it hard to decort; therefore, maintained as whole grains it provides a better source of fatty acids than refined ones (Gebremariam *et al.,* 2014a; Kaleab 2014).

2.5.1.3 Fibre

The crude fibre content of tef (3.0g/100g) which is higher than most gluten-containing and gluten-free cereals. Tef is regarded as a whole grain cereal, and whole grain cereals have higher fibre content than cereals that have undergone dehulling. Another reason would be that small grains have relatively high proportions of bran, which has higher fibre content. The dietary fibre content of tef (8.0g/100g) which is higher when compared to some fruits, nuts, pulses and cereal such as corn and rice (Gebremariam *et al.,* 2014a)

2.5.1.4 Minerals

Tef can be considered a mineral powerhouse when compared to the other cereals. Tef is rich in minerals, namely calcium, zinc, magnesium, iron, phosphorus, and copper. Bultosa (2007) reported that the content of 13 varieties ranges between 3.16-1.99 % with a mean of 2.45 % and a zinc content that is higher than that of sorghum and wheat. The mineral content in tef grains varies between and within tef varieties. Red tef has a higher iron and calcium content than mixed or white tef (Abebea *et al.,* 2003; Kaleab 2014). In other cases, the white tef has a higher copper content than red and mixed tef.

The mineral content of tef has been questioned due to the high iron content, and this has been attributed to soil contamination. Hallberg (1974) investigated the iron content of tef compared with other cereals. When the grains were washed with diluted hydrochloric acid, the iron content dropped from 39.7mg/ 100g to 3.5mg/100g and concluded that the iron content of tef is no different from that of other cereals. When comparing uncontaminated tef to barley, wheat, maize and sorghum, Mengesha (1996) found tef superior in its mineral content, particularly in calcium and iron. Tef grains are quite small, and the increased contact time with soil over a large area results in its mineral contamination. In Ethiopia, tef grains are allied to the traditional threshing of grain under cattle hooves. Bachewe *et al.* (2015) compared the iron content of tef grains that were threshed in the laboratory (manually) and those traditionally threshed. Traditionally threshed seeds had the highest iron content 30-38mg/100g, implying that although the intrinsic iron content of tef may not be as high as previously thought, tef is a better source of iron than other cereals like wheat, sorghum, barley and maize (Ketema 1997).

2.5.1.5 Vitamins

Tef contains good levels of certain vitamins such as vitamins C (188mg/ 100g) niacin (2.5mg), vitamin A, retinol equivalent (RE) riboflavin (0.2mg) and the thiamine (0.3mg) all per 100g of grain. Thiamine in tef is typically lower compared to that of wheat (0.43mg) and barley (0.38mg) (Gebremariam *et al.,* 2014a).

2.5.1.6 Polyphenols

Tef is assumed to contain a large amount of phenols like millets. Ferulic acid (285µg/g) is the major phenolic compound found in tef. Other phenolic compounds found in tef include protocatechuic (25.5µg/g), gastric (15µg/g), vanillic (54.8µg/g) syringie (14.9µg/g) coumaric (36.9µg/g) and cinnamic (46µg/g) (Ketema 1997; Kaleab 2014).

Table 1: Amino acid, fatty acid and macro composition of tef grain compared to maize, sorghum, wheat and rice

	TEF	MAIZE	SORGHUM	WHEAT	RICE
Amino acid (g / 16 g N)					
Lysine	3.7		0.3	2.1	3.7
Isoleucine	4.1		0.7	3.7	4.5
Leucine	8.5		2.1	7.0	8.2
Valine	5.5		0.8	4.1	6.0
Phenylalanine	5.7		0.9	4.9	5.5
Tyrosine	3.8		0.7	2.3	5.2
Tryptophan	1.3		0.2	1.1	1.2
Threonine	4.3		0.5	2.7	3.7
Histidine	3.2		0.4	2.1	2.3
Arginine	5.2		0.6	3.5	8.5
Methionine	4.1		0.3	1.5	2.7
Cystine	2.5		0.3	2.4	1.8
Asparagine	6.4			5.1	9.0
Serine	4.1		0.8	5.0	5.0
Glutamine + Glutamic Acid	21.8			29.5	17.0
Proline	8.2		1.3	10.2	5.0
Glycine	3.1		0.5	4.0	4.5
Energy (kcal)	357	375	370	359	357
Starch (%)	73	72	63	71	64
Crude protein (%)	11	8-11	8.3	11.7	7.3
Linoleic acid (LA)	0.9	1.7	1.3	0.5	0.78
α-linoleic acid (ALA)	0.14	0.05	0.07	0.03	0.03
LA:ALA ratio	7:1	34:1	20:1	17:1	26:1
Crude fibre (%)	3.0	-	0.6	2.0	0.6-1.0
Total dietary fibre	4.5	2.6			-
Soluble dietary fibre	0.9	0.6			-
Ash (%)	2.8	1.4	1.6	1.6	1.4

2.6 UTILISATION OF TEF

Local Ethiopian farmers mostly obtain their income from cultivating tef, generating approximately 500 million USD income per year for local farmers. Agriculture represents 41% of Ethiopia's GDP overall contributing to food security (*Working Strategy for Strengthening Ethiopia's Tef value chain* 2013). Tef makes up 20% of all cultivated area in Ethiopia, covering a staggering 2.7 million hectares with approximately 6.3 billion farms in 2011/2012. Tef might be the most important crop in Ethiopia, but over time, a decline in yields has been observed, and this is due to different economic factors and agricultural factors (Minten *et al.,* 2013).

Tef might be a small-sized cereal but its nutritionally dense. Its an investment crop, not only can it be grown in waterlogged areas, but it can also withstand anaerobic conditions better than other cereals such as maize, wheat, and sorghum (Ketema 1997). Tef is a relatively low risk and reliable crop. It is not attacked by storage pests or weevils which results in reduced post-harvest management costs. The introduction of tef to other parts of the world could be beneficial not only as a forage crop but also for cultivation. The prospects of the crop outweigh the limitations, which include sowing and threshing due to its small size (Ketema 1997; Belay *et al.,* 2008).

Tef is grown predominantly in Ethiopia as a cereal crop rather than as an animal feed. Tef grains are ground into flour to produce whole wheat flour which is used for baking, porridge making and local alcoholic drinks called tela and katikal (Umeta and Faulks 1988). Tef flour is primarily used to make Ethiopian staple foods, including a pancake-like fermented flat bread which is characterised by honeycomb eyes (Figure 7 and Figure 7a). The traditional name is tef-injera, and it is consumed with every stew, replacing rice and bread. Tef grains being the preferred cereal for injera production contribute to its favoured sensory attributes such as the colour, aroma and its ability to be rolled without cracking (texture) is an important quality since it allows for easy wrapping of the sauces (wot) consumed with it. Various studies (Zegeye 1997; Yetneberk *et al.,*

2004) showed that tef grain appeared superior among other cereals grains because of its high resistance to stalling. During the baking of injera, starch is completely gelatinised to form a steam-leavened, spongy matrix in which fragments of bran, embryo, microorganisms and organelles are embedded together (Bultosa and Taylorb 2004). Tef straws are used to feed cattle and also used to reinforce mud and plaster walls of turkuls and local grain storage facilities called goten tef grain. Tef mineral content is well balanced and has been incorporated into baby foods along with soya beans, chickpea (Minten *et al.,* 2013).

2.7 TEF-INJERA FERMENTATION

Tef-injera is made just like any sourdough, by mixing the flour and water at a ratio of 1:2 and allowing it to ferment naturally for two to three days depending on the ambient temperatures (Ashenafi 2006). In the early hours of the fermentation, there is vigorous gas evolution and maximum dough rising, which is followed by a yellow acidic liquid that is formed on top of the dough. The yellow liquid that has formed on top of the batter is discarded after 30-33 hours and replaced with fresh water (Attuquayefio 2014; Mezemir 2015). Back slopping is also practised in the making of tef-injera, the yellow liquid is sometimes not discarded but kept for use as inoculum for the next fermentation (Abiyu *et al.,* 2013; Fischer *et al.,* 2014).

Once the yellow acidic liquid has been removed, the first stage of fermentation concludes, at this time, the pH and gas production decreases as a result. An absit is prepared by mixing 10% of the batter with three parts of water which is allowed to gelatinise for 2-5 minutes. Once it has cooled to 50-55˚C, it is mixed back to the original batter and finally left to ferment for 30 minutes or two hours depending on the textural output being desired. The final pH of the batter is normally 4 due to the action of LAB (Gashe 1985). Absit ensures that desired textural characteristic results after

baking because injera baked without absit or less absit results in injera without eyes, which is a desirable characteristic (Ashenafi 1994; Mezemir 2015). The honey comb like eyes or pores assist in soaking the stews that are consumed with injera (Zegeye 1997).

Absit gives the batter the right viscosity, which contributes to its ability to bend without breaking when picking up stews. Attuquayefio (2014) showed that fermentation time and viscosity play a vital role in the formation of desirable characteristics such as eyes and elasticity as a batter of low viscosity has fewer eye formations compared to batter with a high viscosity. During the fermentation of tef, carbon dioxide is produced, which also gives injera its desirable characteristics. The elasticity of the finished product is said to be the result of proteins during the fermentation period (Attuquayefio 2014). Once the fermentation process is completed, the batter is poured onto a clay plate known as *mitad* with a 45-60cm diameter and is baked for two to three minutes at 200˚C (Refera 2001; Mezemir 2015). The mitad should be cleaned prior to baking with a piece of cloth after greasing with kale and cotton seed; this ensures that the batter does not stick to the mitad (Ashenafi, 2006).

Lize (2017) master's thesis focused on food security and the importance of tef in Addis Ababa. One of the surveys conducted revealed that injera is on the menu for most of the households. Tef is consumed multiple times a day, by both low- and high- income groups. Figures 8 and 9 show that 98.9% of the population consumes more injera than any other product group. The preference of injera over other food products is because of its nutritional value, cultural value, social value and also its good taste. It is evident that injera is the most important food item prepared from tef and is consumed with spicy stews made from meat, beans, dairy products or cabbage. The consumption of injera by the vast population of Ethiopians has been shown to reduce the iron deficiency in Ethiopians. (Molineaux and Biru 1965) reported that non-tef consumers have lower

levels of haemoglobin. Tef consumers have higher levels of haemoglobin, therefore, they do not develop hookworm anaemia if they are infested with hookworm,(Assefa *et al.,* 2013)

Figure 7: Ethiopian injera traditionally prepared from ivory (white tef seeds) and brown tef seed (Laki 2019a)

Figure 7a: Injera made from barley flour (Laki 2019b)

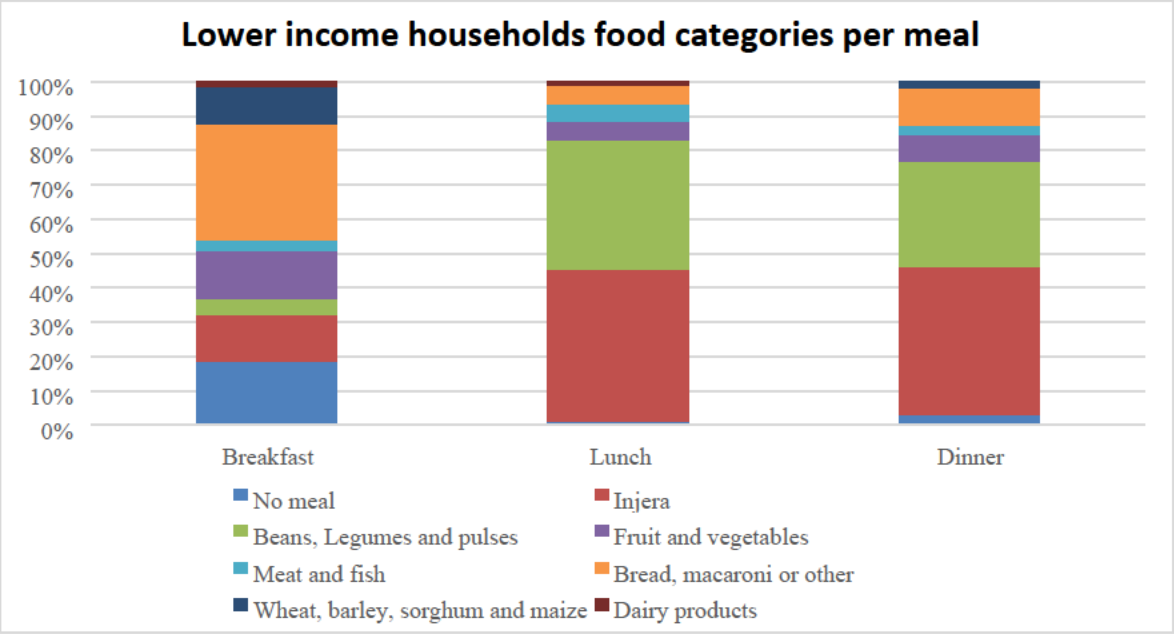

Figure 8: lower income household food categories per meal (Lize 2017)

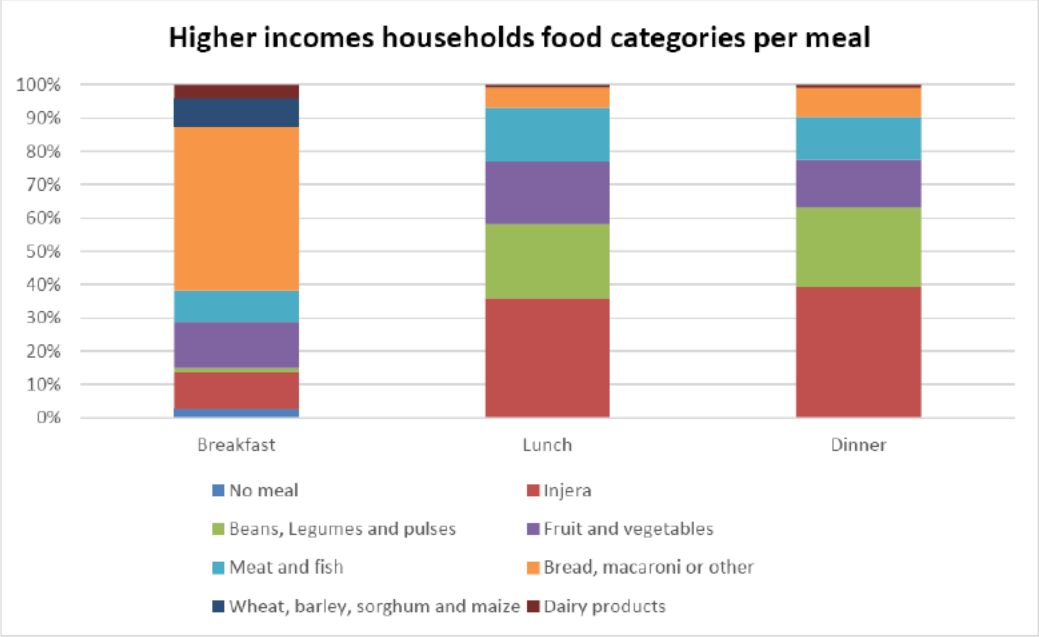

Figure 9: Higher income household food categories per meal (Lize 2017)

2.8 HEALTH BENEFITS OF TEF SEEDS AND TEF BASED PRODUCTS

2.8.1 IRON DEFICIENCY

Iron deficiency is the widest spread micronutrient deficiency globally, a burden apparent in third world countries or developing countries where a high number of infants, young children, and pregnant women are mostly affected and infected. The effects of iron deficiency are very adverse, and they include growth retardation, impaired mental and psychomotor development, child and mental mobility and mortality and decreased immunity and work performance (Belay *et al.,* 2008; Kaleab 2014). Effective measurements or strategies can be employed to prevent and reduce cases of iron deficiency, such as fortifying food and nutritional supplements. Tef can be used because it contains an excellent amount of minerals. Previous studies had shown that when wheat bread was supplemented with 30 % tef flour, the iron content of resulting flour doubled (Bokhari *et al.,* 2012). Therefore, an average daily consumption of 200g of tef enriched bread provides between 42-81% and 72- 138 % of daily intake requirements for iron in women and men. The bioavailability of iron in tef varies depending on the type of treatment, during the fermentation of injera. A significant decrease in phytate content results in an ideal phytate to iron molar ratio (Umeta *et al.,* 2005). Bokhari *et al.* (2012) showed that consuming 30 % of tef enriched wheat bread helped maintain serum iron levels in pregnant women. The study also suggested that the degradation of phytates may lead to better iron bioavailability. There is a need to investigate further the iron bioavailability of tef from the previous studies carried out since Tef has a great potential to improve the nutrition of foods with low iron content.

2.8.2 CELIAC DISEASE

Celiac disease was once considered a gastrointestinal disease affecting mainly Caucasians and is now noted as a systemic disease that may affect many persons at any age, race and ethnic groups. Celiac disease is an auto-immune disorder caused by the assimilation of gluten in inherently

predisposed individuals and occurs in genetically susceptible individuals that carry either Human leukocyte antigen (HLA-DQ2) subtype or DQ8 molecules (Gujral *et al.,* 2012; Spijkerman *et al.,* 2016). Cereals that contain T-cell-stimulatory peptides are gluten-containing cereals such as wheat, barley and rye, which triggers an immune disorder with those individuals who genetically carry the HLA-DQ2 or DQ8 molecules. Celiac affected individuals may exhibit gastrointestinal symptoms, extra-intestinal or no symptoms; classical symptoms include gastrointestinal-related symptoms such as diarrhoea, steatorrhea and weight loss due malabsorption and extra-intestinal or atypical symptoms such as anaemia, osteoporosis, dermatitis herpetiformis, neurological problems and dental enamel hypoplasia (Tjon *et al.,* 2010; Gujral *et al.,* 2012). The only treatment known and accepted for celiac disease is medical treatment therapy and maintaining a strictly gluten-free diet (Kaleab 2014). A study carried out at the Leiden University in the Netherlands, analysed 13 varieties of tef and wheat, barley, oats, maize, triticale and rice as control. A T-cell and antibody assay was carried out by Liesbeth Spaenij-Dekking *et al.* (2005) on different cereals to detect the presence of T-cells stimulatory epitopes of α-gliadin, γ-gliadin and low molecular and high molecular weighted glutenin. Results showed that all tef varieties lacked the T-cell stimulatory epitopes and detected for all known gluten-containing cereals. These findings indicate that tef may be suitable for use in the diet of celiac patients, but more studies could be carried out on whether tef is safe for celiac patients because of limits of currently available methods (Liesbeth Spaenij-Dekking *et al.,* 2005). The mineral content, fibre and phytochemicals and its gluten-free status make it a better cereal to consume than other gluten-free and pseudo-cereals such as quinoa, amaranth, buckwheat and sorghum (Kaleab 2014).

2.8.3 DIABETES

Tef is consumed as a whole grain because of its extremely small size and difficulty of dehulling. Consuming a diet high in whole grain is associated with 20-30% reduction in the risk of developing type 2 diabetes since tef is consumed as a whole grain; therefore, a similar effect can be expected (De Moura 2008). The type of carbohydrates and the rate of digestibility play a crucial role in the glucose level. Tef seeds are mainly 73% complex carbohydrates, which means they are slowly broken down by the digestion system (Kaleab 2014). Relative to wheat, tef has a low glycemic index and thus a much better suit for diabetic patients. The high fibre content in the tef relative to other common cereals decrease fasting blood glucose levels and thus, contributes to the prevention and management of diabetes (Gebremariam *et al.*, 2014b).

2.9 BAMBARA GROUNDNUTS (*VIGNA SUBTERRANEAN)*

Legumes have long been serving as an inexpensive source of non-processed proteins in rural and urban areas. They have major potential in the fight against malnutrition and poverty. Legumes are a good source of fibre, resistant starch, and other nutrients (Bamshaiye *et al.,* 2011).

Voandzeia subterranean (L.) thousars, synonyms of *Vigna subterranean* are commonly known as Bambara groundnuts (figure 10). The bean-like seeds are dug out from the ground like peanuts. The seed-like bean belongs to the Plantae, family of Fabaceae and the subfamily of Faboidea. Bambara groundnuts are rounder than peanuts with an inside seed shaped more like peas. The legume is said to be traced back to Sahelian in the Western part of Africa, and its name originates from the Malawian Bambara tribe (Mubaiwa *et al.,* 2017). Bambara groundnuts can tolerate very dry conditions and soil pH between 5.0- 6.5, serving greatly in regions where rainfall is below 500mm per annual and where rainfall is greater than 500mm per annual it still thrives (Olaleye *et al.,* 2013). Bambara groundnut does not only thrive in different climatic conditions, but its hard coat prevents it from insect attacks; therefore, prolonging its shelf life (Brough *et al.,* 1993b)

Bambara groundnuts, which originated in Africa, was ranked as the third most important crop in Africa, after cowpeas. The legume is mostly grown throughout Africa but widely grown and utilised in Nigeria with a thousand metric tons produced annually. Bambara groundnuts are also cultivated in some parts of Asia, Northern Austria, central and South America (Bamshaiye *et al.,* 2011; Diedericks and Jideani 2015). It is cultivated by farmers as a famine culture crop because of its agronomic values and ability to produce in soil not suited for the cultivation of crops or related common beans and groundnuts. The legume is mostly cultivated by women providing income and daily meals for most households (Brough *et al.,* 1993a; Olaleye *et al.,* 2013). Even though it is

grown in most African countries, the legume is still underutilised. In recent years the yield of Bambara groundnuts production has been low and not investigated by scientists in regards to its functional properties (Bamshaiye *et al.,* 2011).

A

B

Figure 10: (A) Varieties of South African Bambara groundnut and (B) cream white Bambara groundnuts

2.9.1 NUTRITIONAL COMPOSITION OF BAMBARA GROUNDNUTS

Bambara groundnuts are regarded as a balanced food because it is rich in iron and essential amino acids such as lysine and relatively low sulphur-containing amino acids such as methionine and cysteine (Brough *et al.*, 1993b). The proteins contain both essential and non-essential amino acid at 32.7 and 66% respectively. The main amino acid being lysine, which is about 10.3% of the total amino acid content of the seed (Mohammed *et al.,* 2009; Mohammed *et al.*, 2016).

The legume has a great potential to improve malnutrition and food security, as it is inexpensive, easily stored and transported non-processed protein (Baryeh 2001). Nutritionally Bambara groundnuts are rich in iron 4.9-48mg/100g, proteins 18-24%, ash 3.0-5.0%, fat 5.0-7.0%, fibre 5.0-12%, potassium 1144-1935mg/100g, sodium 2.9-12.0mg/ 100g, calcium 95.8-99mg/ 100g, carbohydrates 51-70%, oil 6-12% and energy 367-444Kcal/ 100g (Abdualrahman *et al.*, 2012). The energy value of Bambara groundnuts is greater than that of cowpea and pigeon pea; in addition

to that, it has been reported to contain high levels of carbohydrates (63%), oil (18%) and fatty acids (linoleic, palmitic and linolenic acid). Compared to other legumes, Bambara has a far better concentration of soluble fibre (Olaleye *et al.,* 2013). The dietary fibre of Bambara groundnut compared to other legumes is good and, it is classified amongst the important ingredients to be included in everyday diets (Yusuf *et al.,* 2008). The health benefits associated with increased intake of dietary fibre include an increased bile acid faecal loss, laxation, blood-cholesterol and glucose attenuation and can lead to a reduced risk of development of several diseases such as diabetes, coronary heart disease, obesity and some forms of cancer. The daily recommended fibre intake ranges from 30-40mg/g (Diedericks and Jideani 2015)

Diedericks and Jideani (2015) recent study revealed that Bambara groundnuts are a good source of insoluble dietary fibre. Insoluble fibre from four varieties of Bambara groundnuts was successfully extracted and characterised for total dietary fibre content, of the four varieties, red (24.3± 1.4 % dry matter) and black eye (23.9± 0.3%) were significantly higher in total dietary fibre compared to brown eye (17.7± 0.7%). Insoluble fibre from the four varieties successfully extracted, brown eye (37.4±1.6%) and black eye (39.4± 1.6%) of Bambara groundnut, insoluble dietary fibre had the lowest yield whereas red eye (48.3±1.0%) had the highest insoluble dietary fibre yield. Swelling ranged from 6.37 to 7.72 ml/g. The swelling capacity of brown was (7.72±0.49ml/g) (Wang and Toews 2011).

Bambara groundnuts seeds are consumed in various forms. They can be boiled to make stews and sweetened to prepare a pudding and widely used to make indigenous bread or to make milk similar

to soya milk. Mohammed *et al.* (2016) and Abdualrahman *et al.* (2012) reported Bambara flour in Sudan being used to improve the nutritional value of wheat bread.

The difficulty of converting this legume to edible stews and other various forms is a major problem in rural areas as it takes a long period for it to be softened, an overlooked phenomenon, which is the reason for its decline in use. In rural parts where energy sources are firewood, it is a big hassle to prepare Bambara groundnuts. Mubaiwa *et al.* (2017) arrayed some of the expected challenges during processing leading to the hard to cook phenomena, the seed coat's thickness, porosity, germ length, width to mention a few, play a role in the processing of Bambara groundnut. The manner at which legumes are stored contributes to this type of phenomena. Hot and humid conditions result in the hardening of the seed; therefore, more than normal cooking time and energy are required. Hardening of legumes is said to be attributed to pectin-phytate and the lignitation mechanisms which occur at high temperatures and humidity as suggested by Aguilera and Stanley (1985). Suggested means of managing the hard to cook phenomena include storing legumes at low temperatures, soaking legumes in salt solution before cooking, germinating, milling, roasting, canning, fermenting legumes (Mubaiwa *et al.*, 2017).

Bambara groundnuts also have great potential for use in the food industry as a functional food or ingredient. A study carried by Oyeyinka *et al.* (2017b) on the physicochemical properties of starches extracted from three Bambara groundnut landraces revealed that the extracted starch of all types exhibited thickening properties. Within the types, starch extracted, the gelatinization temperature and amylose content were different, which could be due to the type of land but exhibited oval-shaped and A-type crystals crystalline pattern. Bambara groundnuts contain fairly high resistant starch for use in the food industry (Oyeyinka *et al.*, 2016).

2.9.1.1 Anti-nutrients compound in Bambara groundnuts

Anti-nutrients such as trypsin inhibitors and condensed tannins limit the bioavailability and digestion of nutrients in the Bambara seed for animal and humans. These condensed tannins are polyphenolic substances distributed over the plant, more especially in legumes. Protein digestibility is inhibited by the condensed tannin's large structure by forming irreversible complexes with proteins (Bamshaiye *et al.*, 2011).

Trypsin inhibitor is another anti-nutrient which inhibits the activity of digestive enzymes. Trypsin inhibitor activity in raw Bambara groundnut varieties was shown to be 9.4 Tiu/mg protein and 12.2 Tiu/mg protein (Apata and Ologhobo 1997). Trypsin inhibitors act by inhibiting the activity of digestive enzymes and, it can be inactivated by autoclaving, although the trypsin activity remained after the treatment (Bamshaiye *et al.*, 2011). Trypsin activity varies depending on the genotype, environmental conditions such as fertility, climate seasonality, rainfall, and light intensity. Effective utilisation of proteins, carbohydrates, and minerals depend on the removal of anti-nutrients (Olaleye *et al.*, 2013).

Condensed tannins are polyphenolic substances widely distributed in plants especially in legumes, and due to their large structure, are known to inhibit protein digestibility by forming irreversible complexes with proteins, thereby reducing the bioavailability of amino acids, even though some research has shown that condensed tannins in low concentration have some beneficial effects on humans and animals (Akindahunsi and Salawu 2005). Condensed tannins content was found to range from 0.37 to 0.39% in two Bambara groundnut Landraces. Tannin content can be reduced by different processing methods, such as cooking and roasting.

2.9.1.2 Market potential of Bambara groundnuts

Bambara groundnuts are of a high cost compared to competing products such as cowpea (Vigna unguiculata) and beans (Phaseolus vulgaris). This could be possible due to the low production

output, as Bambara groundnut is known as a famine crop, mainly grown by women on small, substantial farms. The lack of promotion of Bambara groundnut and little investment in the development of functional products has led to the lack of demand and production of Bambara groundnuts (Mkandawire 2007).

Consumer awareness and acceptability studies were carried out in rural parts of KwaZulu-Natal on Bambara groundnut as a potential source for use in complementary foods. In the study by Oyeyinka *et al.* (2017a) Bambara groundnut puree was comparable to reference bean puree and a significant difference ($p<0.05$) was observed between Bambara groundnut samples in terms of aroma, colour and overall acceptability, indicating a preference for the brown Bambara groundnut variety.

Chapter 3

Identification and screening of lactic acid bacteria and yeast during the fermentation of tef-injera and injera fortified with Bambara flour using molecular-based techniques

Abstract

In this chapter, lactic acid bacteria and yeast species from lab fermented tef injera and injera fortified with Bambara groundnut were identified and characterised using culture-dependent and independent techniques. Tef-injera made from only tef flour and the newly developed injera like-product, which contains 12% Bambara groundnuts flour, were spontaneously fermented in the lab following the traditional fermentation method which lasts 72 hours. Samples were drawn at 12 hours interval, and a total of 70 LAB isolates and 30 yeast isolates were identified from both fermentations using rep-PCR fingerprinting followed by sequencing the 16S rRNA gene and the D1/D2 region of the 26S rRNA gene. Cluster analysis of DNA fingerprints of isolates was performed using Bionumercis 7.0. *Weissella confusa* predominated at 24 h in traditional tef-injera fermentation and *Lactococcus lactis* and *Lactobacillus curvatus* predominating at 48 h and 72 h respectively. During the spontaneous fermentation of injera fortified with Bambara flour, Enterobacteriaceae predominated the beginning of the fermentation with *Lactococcus lactis* and *Weissella confusa* dominating 24-72 h. Yeast isolates identified in both fermentations were non-fermenting yeast *Cryptococcus* being the dominating yeast species.The unambiguous identification of LAB and yeast from tef-injera and injera fortified with Bambara flour using molecular biology-based methods is the first step in the future development of defined starter cultures as a feasible means of optimising the fermentation of tef-injera and injera fortified with Bambara flour.

Keywords**:** Injera, rep-PCR fingerprinting, Lactic acid bacteria, yeast

3.1 INTRODUCTION

Injera, a fermented pancake-like sour bread is the most common staple food for a majority of Ethiopians (Abiyu *et al.,* 2013). It is mainly prepared from tef flour (*Eragrostis tef),* an ancient cereal, indigenous to Ethiopia. Tef is gluten-free and well known for its high content of micronutrients such as Fe, Ca, Zinc, B_1 vitamin and is also a rich source of fibre (Ashenafi 2002; Shumoy *et al.,* 2017). However, the protein content of tef (9-11%) is rather low, like other cereals. Improving its protein content can be achieved by supplementing with inexpensive, protein-rich legumes such as Bambara groundnuts *(Vigna subterranean)* which have a higher protein content ranging between (18-24%) and high levels of lysine (6.5-6.8%) and methionine (1.8-2.84%) (Abdualrahman *et al.,* 2012). Injera fortified with Bambara groundnut flour was made by substituting Bambara flour to tef flour at 9% and 12%, for this study only injera substituted with the highest percent was analysed. The fermentation process of tef-injera lasts two-three days, depending on the ambient temperatures (Ashagrie and Abate 2012; Tesfay *et al.,* 2014). The long fermentation time needed for tef-injera fermentation makes the process relatively ineffective. With the increased demand for fresh tef-injera, the fermentation period is not cost-effective for restaurant owners and women running households; therefore, an optimised, and faster fermentation process is needed. The use of starter cultures will not only speed up the fermentation process, but it can lead to a cereal fermented product with desirable functional characteristics, such as increased bioavailability of essential amino acids, minerals and vitamins (Achi 2005; Ogunremi *et al.,* 2017).

The long fermentation time involved in the preparation of tef-injera and injera fortified with Bambara flour can be overcome by using defined starter cultures. However, the idea of starter culture application as a means of optimising tef-injera and injera fortified with Bambara flour fermentation can only be realised fully when the studies have been conducted to identify the

microorganisms involved in the fermentation process, to give a better understanding of the fermentation (Nalbantoglu *et al.,* 2014).

In previous studies, lactic acid bacteria and yeast were reported as the dominating microorganisms during the spontaneous fermentation of injera (Ashenafi 2006). In these studies *Pediococcus pentosaceus*, *Lactobacillus fermentum*, *Lactococcus piscium*, *Lactococcus plantarum* were identified as the responsible LAB species and the yeast species comprised of *Saccharomyces cerevisiae*, *Candida humilis*, *Candida tropicalis*, *Saccharomyces exiguus* and *Pichia norvegensis* were identified as the dominating yeast species (Desiye and Abegaz 2013; Mulaw and Tesfaye 2017). The above-reported studies, only made use of phenotypic methods with low discriminative power and poor reproductivity were applied to determine the microbial diversity and succession in injera. Changes in taxonomical status of some microbial species and discovery of many new LAB and yeast species, identification of microorganisms using molecular tools for ambiguous determination of microbial diversity to obtain a more comprehensive understanding of the microbial ecology (Guyot 2010).

Tilahun *et al.* (2018b) investigated the lactic acid bacteria in commercial tef-injera by sequencing the 16S rRNA gene of isolates and reported *Lactobacillus paracase*i, *Lactobacillus brevis*, *Enterococcus durans*, *Enterococcus hirae Enterococcus avium* and *Enterococcus faecium* as lactic acid bacteria responsible for the fermentation. Despite that, there is a need for a combined analysis of LAB and yeast involved in the spontaneous fermentation of injera using molecular techniques to obtain a more comprehensive understanding of the microbial ecology and succession taking place in injera processing. Combining typing-methods such as repetitive extragenic palindromic-PCR (rep-PCR) and sequencing the 16S rRNA gene or the 26S rRNA gene offer rather rapid

identification and characterisation of microorganisms from different types of food sources compared to only culture-based methods (Ruiz *et al.*, 2014).

The aim of this study was to identify and characterise lactic acid bacteria and yeast involved in the spontaneous fermentation of tef-injera and injera fortified with Bambara flour using rep-PCR fingerprinting and sequencing the 16S rRNA gene (for bacteria) and 26S rRNA gene (for yeast). This will provide the foundation for later development of starter cultures suitable for rapid and consistent fermentation of tef-injera and injera fortified with Bambara flour.

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

Tef seeds were purchased from Tef growers South Africa (Pty) Ltd, and cream coated Bambara groundnuts were obtained from Josini Farms, KwaZulu-Natal, South Africa.

3.2.2 PREPARATION OF FLOURS

The flours were prepared according to the a slightly modigied method of Mbata *et al.* (2009). Bambara groundnuts were graded cleaned with water and blanched for 30 minutes to dehull. The dehulled Bambara groundnuts were dried in a conventional oven drier for 48 hours at 45˚C. The dried grains were ground into flour using a Kenwood multi pro grinder and sieved using a 250µm sieve and subsequently kept in airtight plastic bags at room temperature. Tef seeds were ground into flour using a grinder and sieved using a 250 µm sieve and packed in airtight plastic bags for analysis and stored as above

3.2.3 LABORATORY FERMENTATION OF TEF-INJERA AND INJERA FORTIFIED WITH 12% BAMBARA FLOUR

Tef-Injera and fortified injera preparations were performed according to the methods described by Mezemir (2015) and Boka *et al.* (2013) in 900 ml batches. Tef flour (300g) and filtered (0.22µm) water (600 ml) were mixed in sterile 2L Erlenmeyer flasks and incubated at 25±2˚C for 72 hours. After 30 hour of fermentation, a yellow liquid accumulating on the batter was discarded and replaced with filtered water and left to further ferment for 42 hours For injera fortified with 12% Bambara flour, 900 ml batch was prepared by adding 36 g of Bambara flour and 264 g of Tef flour and mixed with 600 ml of filtered water to ferment. Samples were aseptically collected at 0, 24, 48 and 72 hours and kept at -80 ˚C in 20% (v/v) glycerol stock until analysis.

3.2.4 PH DETERMINATION

The pH of the batter from both fermentations was analysed using a pH-meter (PHM82, Radiometer, Brønshøj, Denmark) at 0, 24, 48, and 72 hour.

3.2.5 ENUMERATION AND ISOLATION OF LACTIC ACID BACTERIA (LAB) AND YEAST

Samples were aseptically collected from tef-injera and injera fortified with Bambara flour batter and 1 ml homogenized in 9 ml of 0.9% sterile saline water. Serial dilutions (10^{-1} to 10^{-7}) were performed according to the method of Akabanda *et al.* (2013). For each sample and 0.1 ml of appropriate dilutions was inoculated onto MRS agar plates for enumeration of total lactic acid bacteria (LAB) and Malt Extract agar (MEA) (Merck) containing 100 mg/ml chloramphenicol and 50 mg/l chlortetracycline for enumeration of yeast. MRS plates were incubated anaerobically for three days at 30˚C using anaerobic jars with the Merck anaerocult A GasPack anaerobic system,

while MEA plates were incubated for three days at 25˚C. After incubation, ten colonies from a sector of the highest dilution were further purified by successive streaking on MRS plates for LAB and MEA plates for yeast. For long-term maintenance LAB and yeast isolates were stored at -80˚C in MRS and MEA broths, respectively containing 20% (v/v) glycerol (Akabanda *et al.*, 2013).

3.2.6 PHENOTYPIC CHARACTERISATION OF LAB AND YEAST

Prior to genotypic identification, presumptive LAB isolates on MRS agar were examined for Gram reaction, catalase activity, as well as cell morphology and motility. Gram reaction was performed by dissolving a loop full of freshly grown colony material in a drop of 3% KOH (Merck) on a microscope slide, observing whether ropy strings occurred (Gregersen 1978). Catalase activity was determined by adding a drop of H_2O_2 (Merck) solution (30%) to a colony mass on a glass slide, observing whether air bubbles were generated (Taylor and Achanzar 1972; Nielsen *et al.*, 2007).

3.2.7 DNA EXTRACTION AND GENOTYPIC IDENTIFICATION OF LAB AND YEAST ISOLATES

The 70 MRS and 30 MEA isolates were grouped using Rep-PCR. DNA was extracted using the InstaGen^{TM} DNA extraction kit following the instructions of the manufacturer. The PCR reaction was performed by mixing 3 µl of DNA from pure cultures with 22 µl of PCR mixture, containing PCR master mix (Thermo Fisher Scientific, USA), GTG_5 primer and sterile MilliQ water and were run in SureCycler PCR system (Agilent, USA) using the thermocycling conditions of 95°C for 7 min, 30 cycles of 95°C for 1 min, 45°C for 1 min and 65°C for 8 min, followed by 65°C for 16 min and 4°C overnight. Thereafter, PCR products were visualised in 1.5% agarose gel after 120V, and 2.5 h electrophoresis run (Nielsen *et al.,* 2007). The Rep-PCR profiles were clustered using the Bionumerics V. 7.0 software (Applied Math, Sint-Martens-Latem, Belgium). Calculation of the dendrograms was based on the Dice's coefficient of similarity with the unweighted pair group

method with arithmetic averages clustering algorithm (UPGMA). Representative isolates were selected through the groupings from images of cluster analysis of rep-PCR products, for sequencing of their 16S rRNA (LAB) and D1/D2 region of 26S rRNA (yeast) genes (Jespersen *et al.,* 2005; Akabanda *et al.,* 2013). Before submission for sequencing at the MacroGen (Netherlands), amplification of the 16S genes was done using 27f (5'-AGA GTT TGATYMTGG CTC AG-3') and 1540R (5'-TACGGYTACCTTACGACT-3') primers, and for the D1/D2 region of 26S genes, NL1 and NL4 primers were used, according to the method already described by Nielsen *et al.* (2007). Sequences obtained were filtered and trimmed for alignment using the CLC Genomics Workbench version 11 (QIAGEN Bioinformatics) and then compared with the sequence in the GenBank database using BLAST (NCBI, United States) to obtain the identities of LAB and yeast species (Altschul *et al.,* 1990). The nucleotide sequence obtained in this study has been assigned GenBank Accession numbers, as indicated in Appendix 2.

3.3 RESULTS AND DISCUSSION

3.3.1 MICROBIAL GROWTH AND PH DEVELOPMENT DURING THE FERMENTATION OF TEF-INJERA AND INJERA FORTIFIED WITH BAMBARA FLOUR

The pH of tef-injera decreased from an initial pH of 6.20 to 3.69 at 72 hours (Table 2) at which the fermentation concludes. Fully fermented tef-injera has previously been reported to have a final pH ranging between 4.00-3.88. Tef-injera is a type 1 sourdough, and they are characterised by having a pH of ≈ 4 (Fischer *et al.,* 2014; De Vuyst *et al.,* 2016). The sour taste of injera, which is due to the lactic acid content is a desired characteristic by injera consumers (Mulaw and Tesfaye 2017).

The pH of the batter from injera fortified with Bambara flour showed a similar trend (Table 3.) with a decrease from an initial pH of 6.18 to 4.00 at 72 hours. Lactic acid bacteria count were below the detection limit (<10 CFU/ml) at the beginning of spontaneous fermentation of tef-injera fermentation (Table 2). The rapid growth of LAB was observed in both the tef-injera and injera fortified with Bambara flour fermentation growing well above 8 log (CFU/ml) within 48 hours The high count of LAB for both fermentations, explains the increase in the acidity, as they are known to produce lactic acid as their major product (Salvucci *et al.,* 2016). Unlike LAB, yeast growth was limited, and counts did not exceed 3 log (CFU/ml) for both Injera and injera fortified with Bambara flour fermentations.

3.3.2 INITIAL GROUPING AND IDENTIFICATION OF LAB AND YEAST ISOLATED FROM INJERA AND INJERA FORTIFIED WITH BAMBARA FLOUR

Both LAB and yeast were identified by a combination of phenotypic and molecular methods. Initially, the micro and macro morphological characteristics were recorded. All MRS isolates from tef-injera fermentations were gram positive and catalase negative. Isolates from spontaneously fermented injera fortified with Bambara flour on MRS were both gram positive (77%) and gram negative (23%). Rep-PCR fingerprinting techniques allow typing at sub species level and in some cases reveal significant differences among bacterial strains of the same species from fermented foods (Khemariya *et al.,* 2014). The rep-PCR fingerprinting resulted in six major clusters among the fermented tef-injera and injera fortified with Bambara flour isolates. Sequencing of the 16S rRNA gene identified that the clusters represented *Lactococcus lactis, Lactobacillus curvatus, Weissella confusa, Pediococcus pentosaceus, Enterobacter cloacae* and *Klebsiella veriicola,* respectively (Figure 11 and Tables 2 and 3). For the yeast isolates five clusters were identified representing *Cryptococcus albidosimilis, Cryptococcus chernovii, Cryptococcus albidus,*

Cryptococcus saitoi and *Naganishia friedmannii and Naganishia adeliensis*, respectively (as determined by sequencing of the D1/D2-region of the 26S rRNA gene (Figure 12. and Tables 2 and 3).

3.3.3 LAB DEVELOPMENT DURING INJERA AND INJERA FORTIFIED WITH BAMBARA FLOUR FERMENTATION

LAB dominated throughout the spontaneous fermentation of tef-injera and injera fortified with Bambara flour. As represented in Table 2, during the spontaneous fermentation of tef-injera *W. confusa* predominated at 24 hours and was present until the end of the fermentation, while *Lc. lactis* predominated at 48 hours with *Lb. carvatus* predominating as the fermentation concluded. Homolactic (homofermentative) LAB such as *Pediococcus, Lactococcus,* and some *Lactobacillus* spp. produce lactic acid bacteria as the end product (Ashenafi 2006; Prückler *et al.,* 2015). The later phases (48 hour and onwards) of spontaneous tef-injera fermentation was mostly dominated by homofermentative LABs responsible for reducing the pH of the batter. The obtained results are in agreement with work carried by Ashenafi (2006) who associated tef-injera fermentation with lactic acid bacteria from the *Weissella, Lactobacillus* and *Pediococcus* genera. Desiye and Abegaz (2013) identified *P. pentosaceus, Lactobacillus fermentum* from the beginning to the end of Tef-injera fermentation.

During the fermentation of injera fortified with Bambara flour, *Lc. lactis* was the only LAB isolated at 0 hour (Table 3), while *Klebsiella verricola* and *Enterobacter cloacae* were appearing more abundant constituting 80% of the MRS isolates at 0 hour *Klebsiella* is known to have a fermentative type of metabolism, with glucose being fermented to produce acid and gas. Gashe (1985) reported Enterobacteriaceae as the predominating microorganisms during the first hours of tef-injera fermentation and were able to reduce the pH from 6.6 to 5.8. The presence of

Enterobacteriaceae in the dough has been said to facilitate gas production (Ashenafi 2006). As the pH further decreased to 4.06, the Enterobacteriaceae were outgrown by the LAB and were not detected when the pH reached 4.06 at 48 hour *Lc. lactis* and *W. confusa* predominated at 24 hour and 48 hour of the fermentation respectively.

Spontaneous fermentation of type 1 sourdough normally requires flour, and addition of water and the inoculating microorganisms would mostly originate from the seeds or flour itself (Celano *et al.,* 2016). Ethiopian farmers practice seed threshing, which is carried out on a threshing ground, Awedemma, made by digging out the vegetation inside the soil and smoothing the ground. Tef straws are then threshed by driving cattle over it. This contributes to a type of microflora contaminating the seeds or flour (Assefa *et al.,* 2013; Bachewe *et al.,* 2015). The difference in microbial diversity in Tef-injera can be owed to numerous factors such as the processing of the seeds to the flour type and geographic origin. The nutritional content of flour differs from one geographical region to another, and that affects the sourdough microbiota; especially type 1 sourdough that only requires flour and water to initiate the fermentation and the flour as a source of inoculum (Celano *et al.,* 2016; Gobbetti *et al.,* 2016).

3.3.4 ISOLATION AND IDENTIFICATION OF YEAST FROM TEF-INJERA AND INJERA FORTIFIED WITH BAMBARA FLOUR

Cryptococcus spp. were the most abundant yeast throughout both fermentations, but in all cases in rather low counts. For the spontaneous fermentation of tef-injera, the yeast counts decreased from 3.38 ± 0.04 log (CFU/ml) at 0 hour to 2.93±0.02 log (CFU/ml) at 48 hour, as shown in Table 2. and for injera fortified with Bambara flour fermentation, yeast counts decreased from 2.93 ±0.12 log (CFU/ml) at 0 hour to 2.77± 0.07 log (CFU/ml) at 48 hour (Table 3). Cryptococcus species

were also reported by Tilahun *et al.* (2018a) in Ethiopian fermented injera, other yeast species such as *Saccharomyces cerevisiae, Candida humilis, Candida tropicalis, Saccharomyces exiguous* and *Pichia norvegensis* were also isolated from tef-injera. *Cryptococcus* species do not have the ability to ferment, but they are able to assimilate different sugars (Fell *et al.*, 2001). They may not ferment sugars, but under extreme environmental conditions, such a low pH can produce α-amylases. α-amylase degrades the polyglucoside with α 1,4 glucosidic bonds such as starch and glycogen (Wanderley *et al.*, 2004) and it can be speculated that yeast during the fermentation of Tef-injera, produce amylase at low pH which liberates the starch sugars.

Table 2: Injera type, batter pH and log (CFU/ml) of lacticacid bacteria, yeast and % abundance of yeast and LAB during Tef-injera fermentation (standard deviation in brackets) as determined by enumeration on MRS and MEA, respectively.

100% tef injera	**Fermentation time (hour)**			
	0	24	48	72
pH	6.20	4.19	3.88	3.69
Log (CFU_{LAB}/ml)	BD [a]	8.31 (0.02)	8.57 (0.05)	8.16 (0.06)
% LAB population				
W. confusa		90	20	40
Lc. lactis		10	60	10
Lb. curvatus			20	50
log (CFU_{YEAST}/ml)	3.38 (0.04)	2.61 (0.28)	2.93 (0.02)	[b] No Data
% Yeast population				
Cryp. albidosimilis		60		
Cryp. Chernovii	20	20		
Cryp. albidus	60		60	
Cryp. saitoi	20	20	20	
Nag. Friedmannii			20	

Abbreviations: *W; Weissella, Lc; Lactococcus, Lb; lactobacillus*, Cryp; *Cryptococcus, Nag; Naganishia,*
[a]BD; Below detection limit
[b] No Data, plates overgrown by black mould covering the media

Table 3: Injera type, batter pH and log (CFU/ml) of lactic acid bacteria (LAB), yeast and % abundance of yeast and LAB during the fermentation of injera fortified with Bambara flour (standard deviation in brackets) as determined by enumeration on MRS and MEA, respectively.

Injera fortified Bambara flour	**Fermentation time (hour)**			
	0	24	48	72
pH, batter	6.18	4.16	4.06	4.00
Log (CFU_{LAB}/ml)	5.05	7.43	8.34	8.80
	(0.04)	(0.07)	(0.07)	(0.15)
% LAB population				
W. confusa		20	60	30
Lact. Lactics	20	70	20	60
Pd. Pentosaceus			10	10
% Non-LAB				
Ent. Cloacae	30	10		
Kleb. veriicola	50			
Log (CFU_{YEAST}/ml)	2.81	3.61	2.77	[b] No data
	(0.12)	(0.24)	(0.07)	
% Yeast population				
Cryp. albidosimilis	60			
Cryp. Albidus	40	100	80	
Nag. Adeliensis			20	

Abbreviations: W; *Weissella*, Lc; *Lactococcus*, Lb; *Lactobacillus*, Ent: *Enterobacter*, Kleb: *Klebsiella*, Cryp; *Cryptococcus, Nag; Naganishia*,

[b] No Data, plates overgrown by black mould covering the media

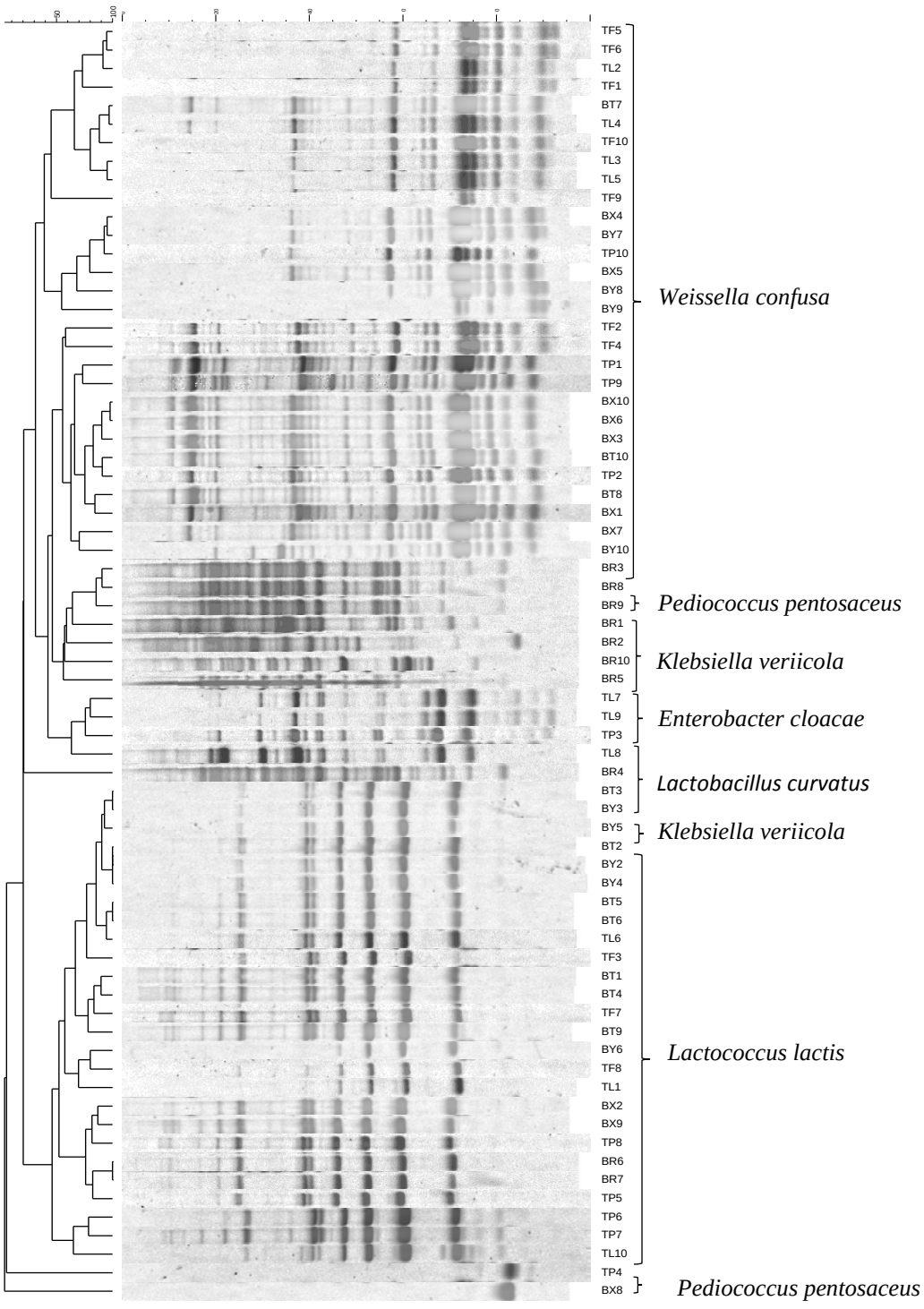

Figure 11: Dendrogram obtained by cluster analysis of rep-PCR (GTG5) fingerprints of LAB isolates originating from tef-injera and injera fortified with Bambara flour. The dendrogram is based on Dice's Coefficient of similarity with unweighted pair group method with arithmetic average clustering algorithm (UPGMA).

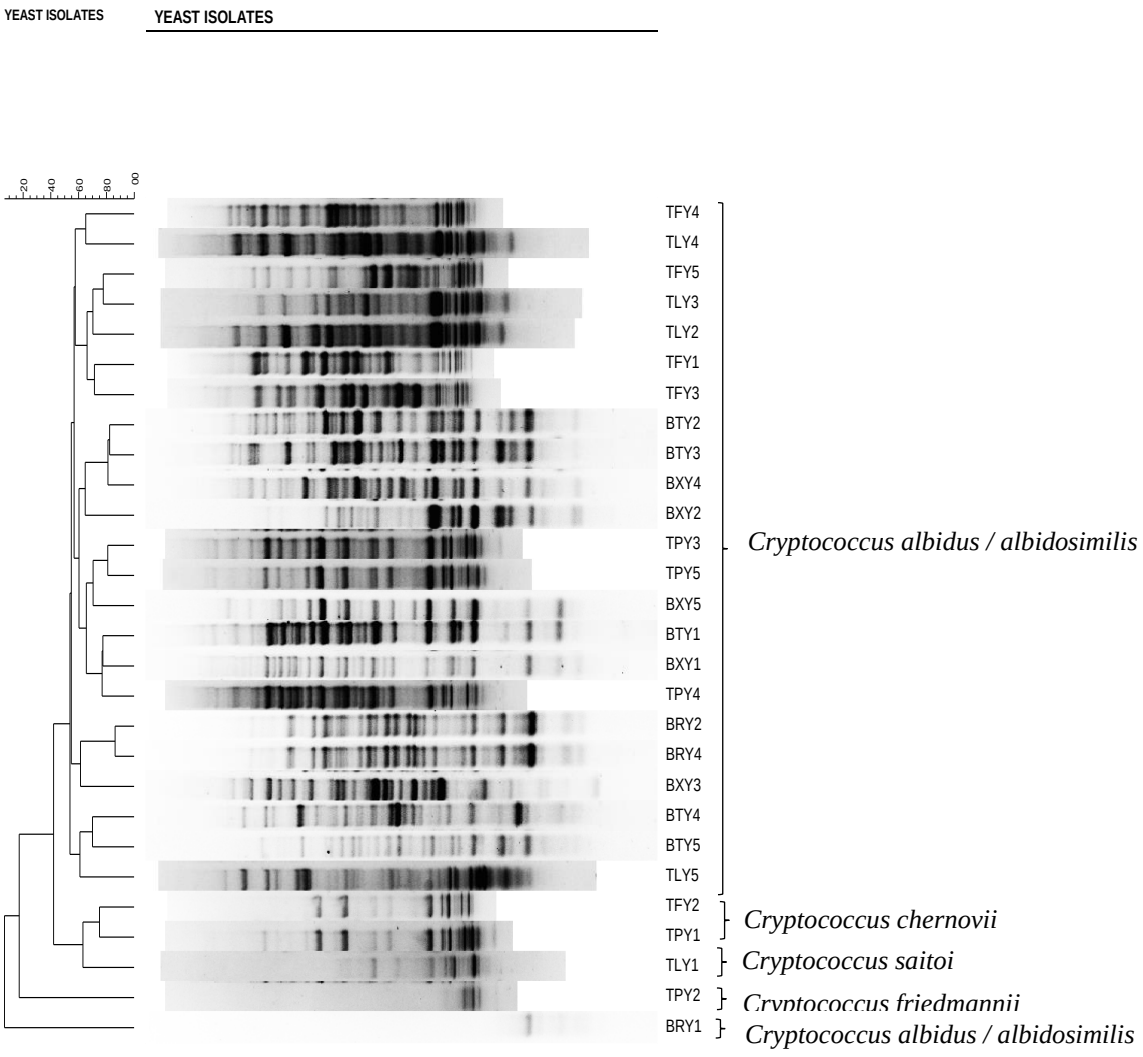

Figure 12: Dendrogram obtianed by cluster analysis of rep-PCR (GTG5) fingerprints of yeast isolates originating from tef-injera and injera fortified with Bambara flour. The dendrogram is based on Dice's Coefficient of similarity with unweighted pair group method with arithmetic average clustering algorithm (UPGMA).

3.4 CONCLUSION

Understanding the microbiology of the fermentation is a prerequisite to develop a suitable starter culture as a measure for optimising the fermentation process of tef-injera and injera fortified with Bambara flour. In this study, LAB and yeast involved in the fermentation of tef-injera and injera fortified with Bambara flour were investigated. LAB were the dominating microorganisms during the fermentation of Injera and injera fortified with Bambara flour with yeast playing a limited role. *Weissella confusa*, *Lc. lactis* and *Lb. curvatus* have the potential of being used as starter cultures for a controlled fermentation of tef-injera and injera fortified with Bambara flour, as they were in Tef-injera and injera fortified with Bambara flour fermentation from the beginning to the end of the fermentation. These isolates have a potential of reducing the pH $\leq$ 4 within 24 hour of the fermentation as they showed good acid production and tolerance; therefore, are good candidates for use as starter culture in tef-injera and injera fortified with Bambara flour fermentation

Chapter 4

Evaluation of lactic acid bacteria starter culture to produce Tef-injera and injera fortified with Bambara groundnuts flour

Abstract

In this chapter, three isolates of lactic acid bacteria *Lactococcus lactis*, *Weissella confuses* and *Lactobacillus curvatus* that were previously isolated from spontaneously fermented Tef injera and injera fortified with 12% Bambara groundnut flour were investigated for their ability to ferment Tef injera and Bambara fortified injera in a combination of 0.1%. The starter culture mixture showed the ability to ferment injera was determined by lowering the pH of Tef injera, injera fortified with 9% Bambara flour and 12% from 5.73 to 3.78, 5.63 to 3.80 and 5.60 to 3.81 respectively within 24 hours of the fermentation. A similar trend was also observed with an increase in titratable acidity of the tef injera and fortified injera samples from 0.26 to 0.92%, 0.25 to 0.79% and 0.22 to 0.83% respectively. Naturally fermented injera and injera fortified samples showed a slower reduction of pH to below 4.00 and a slower increase in titratable acidity after 72 h. A significant difference ($p<0.05$) was observed with Injera fermented using a starter cultures had reached the desired pH and acidity within 24 hours then injera spontaneously fermented. The use of LAB starter cultures improved the fermentation process of injera.

Keywords: Titratable acidity, Lactic acid bacteria, Bambara groundnut

4.1 INTRODUCTION

Tef (*Eragrostis tef*) seeds are an Ethiopian treasure, cultivated as a major cereal and is a staple food for a majority of Ethiopians (Bultosa *et al.,* 2002). Tef seeds are famously utilised to create injera, a fermented sourdough leavened flat bread consumed daily by the Ethiopians and Sudanese. It constitutes 70% of the diet of Ethiopians (Mesfin *et al.,* 2011; Attuquayefio 2014). Tef grains are rich in iron, calcium, magnesium and phosphorus, however they are poor in protein content, and the consumption of tef injera may contribute to protein malnutrition. Injera is usually made with flour from tef grains but can also be made from other grains such as wheat, sorghum and maize (Abiyu *et al.,* 2013). Improving its protein content can be achieved by fortifying with inexpensive, protein-rich legumes such as Bambara groundnuts *(Vigna subterranean)* which have a higher protein content ranging between (18-24%) and high levels of lysine (6.5-6.8%) and methionine (1.8-2.84%) (Abdualrahman *et al.,* 2012). Bambara groundnut flour was substituted at 9 % and 12 % , after pre-trails in the lab and also from trends and previous published work on the enrichment of injera, Girma *et al.* (2013) found that the sensory attribute of injera (rollability, sourness, colour, odour, flavour, injera eyes) were significantly different from the control injera made from tef only. Sensory attribute scores in were high with an increase in the flaxseed substitution levels from 3%, 6% to 9%, scores for rollability, sourness, odour, flavour and a decreasing trend was observed for scores on odour, colour and injera eyes. The overall goal for substituting Bambara groundnut flour into the formulation of injera was to improve the nutritional quality without compromising the desired characteristics of injera, such as injera eyes, odour and flavour, especially since Bambara groundnut have a strong nutty odour and flavour.

As a result, two fortified products were produced by substituting 9% and 12% of Bambara flour to Tef flour. Traditionally injera is made by fermenting tef flour mixed with water (1:2) to naturally ferment or by adding a portion of a previously fermented tef batter to use as inoculum. The fermentation lasts for about 48 to 72 hours, depending on the ambient temperatures. After 72 hours of primary fermentation, 10% by weight of the fermented batter is made into a porridge to form absit. After absit is cooled, it is mixed with the rest of the initial batter to initiate the secondary fermentation which usually lasts for 30 minutes to 2 hours and baked (Ashagrie and Abate 2012; Mezemir 2015). The standard tef-injera has a pH < 4 and titratable acidity of 0.5 -0.9%. The fermenting and dominant microorganisms identified in traditional injera processing are lactic acid bacteria such as *Pediococcus cerevisiae, Lactobacillus brevis, Lactobacillus plantarum* and *Lactobacillus fermentum* (Gashe 1985; Ashenafi 2006; Fischer *et al.*, 2014). Lactic acid bacteria starter cultures have been investigated by Hassen *et al.* (2018) the combination of *L. plantarum and S. cerevisiae* produced better quality rice injera compared to injera produced by the traditional starter (irsho) with respect to appearance, taste, aroma, mouthful and overall acceptability after 24 and 48 hour of fermentation. Processing and controlled fermentation parameters (conditions and formulations) for injera fortified with Bambara groundnut flour and traditional injera are not known, and there is a need to optimise the processing of the innovative product.

4.2 MATERIALS AND METHODS

4.2.1 PREPARATION OF FLOUR

Bambara groundnuts were soaked in water at 1:2 ratio for 48 hours and dehulled then soaked in 1% sodium metabisulphite solution for 5 minutes, rinsed with distilled water and dried for 48 hours

at 45˚C. After which they were grounded into flour, sieved on a 250-micrometer, packed in sterile whirled bags and stored at 4˚ C. Tef grains were soaked in 1% sodium metabisulphite solutions for 5 minutes. They were rinsed in sterile distilled water, dried for 24 hours at 40˚C, ground to powder, sieved and stored at 4˚C until use.

4.2.2 PREPARATION OF STARTER CULTURE

Three LAB species *W. confusa*, *Lc. lactis* and *Lb. curvatus* isolated from tef injera and injera fortified with Bambara groundnut were used for this study. LAB cultures were cultured at 37°C for 48 h on Man-Rogosa-Sharpe (MRS) agar followed by two successive rounds of sub-culturing in MRS broth with incubation at 37°C for 24 and 16 hour, respectively. LAB strains were each added into batter prior to fermentation in cell concentrations of 10^8 cfu/g.

4.2.3 PREPARATION OF INJERA AND INJERA FORTIFIED WITH BAMBARA GROUNDNUT FLOUR FROM LAB STARTER CULTURES.

Fermentation of injera was performed in 900ml batches. Injera containing only tef flour (100% tef injera) and injera substituted with 9% and 12% Bambara flour was prepared, following traditional injera preparation procedure with slight modification (Boka *et al.,* 2013; Mezemir 2015) Injera fortified with 9% Bambara flour was prepared by weighing out 27g of Bambara flour and 273g of tef flour with 600 ml of sterile water and for injera fortified with 12% Bambara flour, 36 g of Bambara flour and 264 g of Tef flour and mixed with 600 ml of sterile water to ferment in a 2L bucket following the same procedure. For injera samples fermented using starter cultres, the flour was mixed with sterile water at respective ratios and LAB cultures (10^8 cfu/ml) at 1% and incubated at 25±2˚C for 48 hours (spoons and flasks used were sterilised). After 48 hours, the absit was prepared by boiling 10% of the fermenting dough and mixing with three parts of water. The absit was then cooled to 50-45˚C and mixed back to the initial dough for the second phase of

fermentation which lasted about 2 hours. The resulting batter was later baked in a heated electric griddle to obtain Tef injera and injera fortified with Bambara flour. The samples were kept in a sealed plastic bag at refrigeration temperature (4˚C) until further analysis was performed.

4.2.4 PH

The pH of the fermented batters was determined directly using a pH meter (CRISON LASEC/SA BASIC 20 pH meter). The pH meter was calibrated using buffer 4,7, and 10 according to the manufacturer's instructions.

4.2.5 TITRATABLE ACIDITY

Ten g of the batter was mixed with 100 ml of distilled water, and five drops of phenolphthalein was added as an indicator and titrated against 0.1N NaOH to a light pink end point and expressed as percent lactic acid (Ough and Amerine 1967).

$$\% \text{ Lactic acid} = \frac{\{ml\ of\ NaOH\ used]\ X\ [0.1N\ NaOH]\ X\ [0.09]}{g\ of\ sample}\ X100$$

4.2.6 STATISTICAL ANALYSIS

Each of the six injera Samples taken for pH and titratable acidity were analysed in triplicate. Mean and standard deviations were calculated, and one-way analysis of variance (ANOVA) was done. Mean separation was done by Tukey post hoc for significant difference amongst means ($p < 0.05$), using IBM SPSS Statistics software.

4.3 RESULTS AND DISCUSSION

4.3.1 CHEMICAL ANALYSIS: PH

The changes in the pH of injera during its spontaneous fermentation shown in Figure 13 and Figure 14 shows pH changes for injera fermented using LAB cultures. The initial pH of unfermented

injera batter at 0 h ranged between 6.4-6.7 (Figure 13). Naturally fermented injera samples in Figure 13 (TIC, 9% TB & 12% TB), the pH remained above 5.0 for the first 24 hour of the fermentation. A significant decrease ($p < 0.05$) in the pH of the naturally fermented injera dropped to below 5.0 from 48 hour, reaching a pH of between 3.8-3.6 72 hour (Figure 13). The pH of the injera samples inoculated with LAB starter cultures is presented in Figure 14. A gradual decline from a pH of 5.8-5.6 to a pH of 4.00 within the first 12 hour of the fermentation noticed. The pH of the samples inoculated with LAB cultures significantly decreased ($p < 0.05$) from a pH between 5.8-5.6 at 0 hour, to 3.8 at 24 hours Hassen *et al.* (2018) also introduced LAB starter cultures and their co-culture yeast cultures, which resulted in a decrease in pH from 6.35 to 4.5 of rice injera within 18-24 hours of fermentation. Injera has been reported by different authors previously to have a pH ranging between 3.65 and 4.02 (Attuquayefio 2014). A decrease in pH results in the increase in acidity, which is a desired characteristic for injera. The addition of starter culture mixture at 1% substantially reduced the fermentation time. Studies of traditional fermented products such as Kunun-zaki (Agarry *et al.,* 2010) Togwa (Mugula *et al.,* 2003) resulted in hastened fermentation.

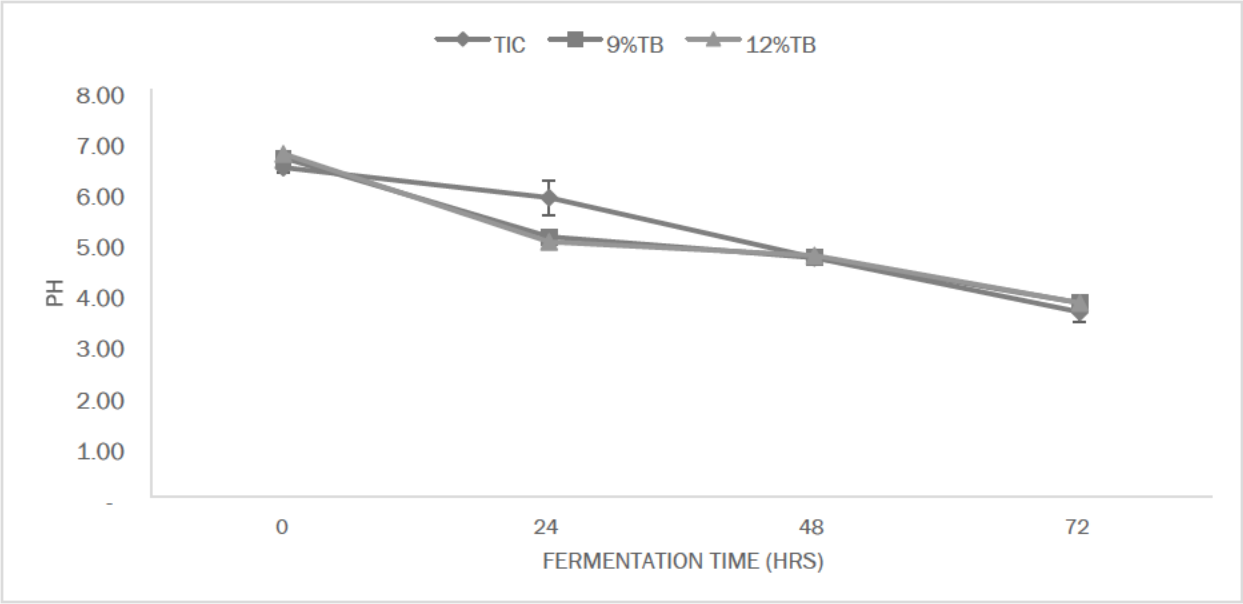

Figure 13: Effect of fermentation time on the pH of injera varieties spontaneously fermented TIC-tefinjera; 9% BT - injera fortified with 9% Bambara flour ; 12% BT- injera fortified with 12% Bambara flour ($p<0.05$)

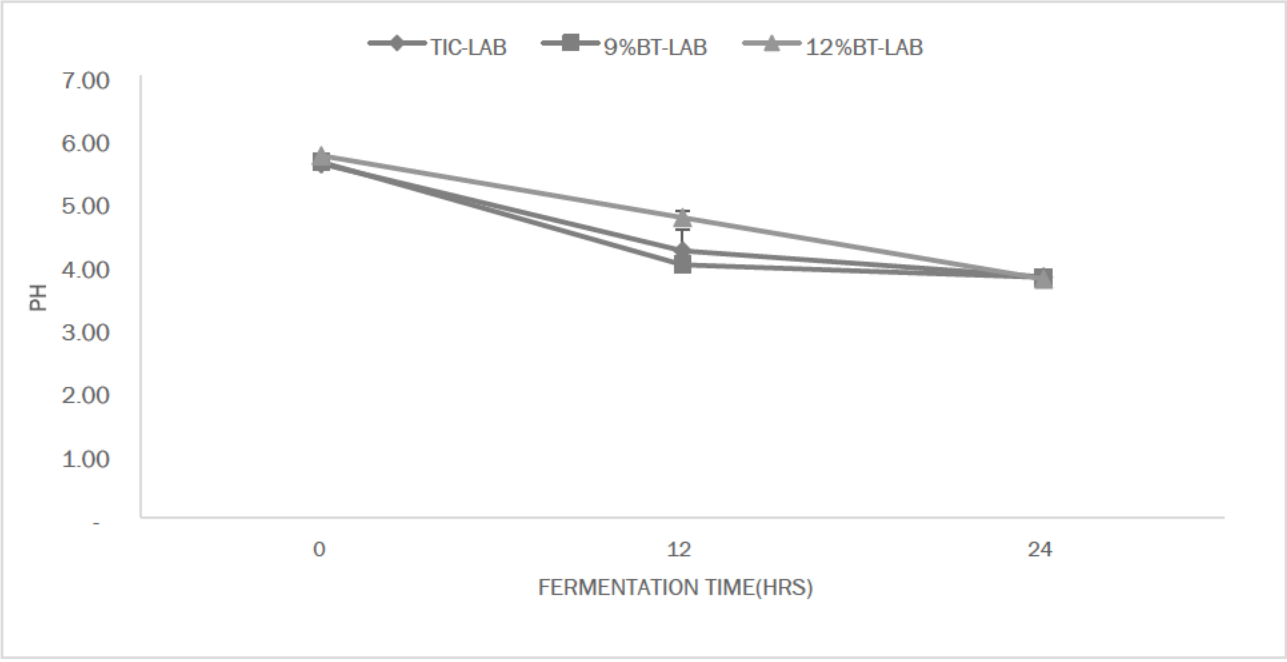

Figure 14: Effect of fermentation time on the pH of injera varieties fermented using LAB starter cultures (TIC-LAB; Tef injera inoculated with LAB cultures, 9%TB-LAB; injera fortified with Bambara groundnut inoculated with LAB cultures, 12%TB-LAB; injera fortified with Bambara groundnut inoculated with LAB cultures ($p< 0.05$).

4.3.2 LACTIC ACID ANALYSIS

Figure 16 and 17 represent the changes in titratable acidity (expressed as % lactic acid) during the fermentation process at 25˚C for injera batter naturally fermented (TIC, 9% TB, 12% TB) and inoculated with LAB cultures (TIC-LAB, 9% TB-LAB, 12% TB-LAB). A general increase in titratable acidity was observed ranging between 0.1-0.2 for unfermented injera batter that were not inoculated with LAB cultures (Figure 15). The amount of lactic acid increased gradually with time, ranging between 0.5-0.7% within 24 hours. At 72 hour a significant increase ($p < 0.05$) was observed in the acidity produced in all samples with TTA values ranging between 1.00- 1.20 was reached. Injera batter inoculated with LAB cultures produced lactic acid faster when compared to the injera batter naturally fermented. Inoculated injera batter samples reached an acidity level ranging between 0.7 -0.9% ($p<0.05$) within 24 hours (Figure 16). *Lactococcus lactis* and *Weissella confusa* are homofermenters which produce more than 90 % lactic acid as their end product (Kandler 1983; Ramaite 2004; Ogunremi *et al.*, 2017), which describes the TTA pattern for the injera samples in Figure 14. Similar observations were recorded for Ting fermented with LAB cultures starter cultures (Ramaite 2004). The addition of LAB starter culture and their co-culture yeast increased the titratable acidity from 0.33% to 0.95% within 18-24 hours of rice injera fermentation (Hassen *et al.,* 2018).

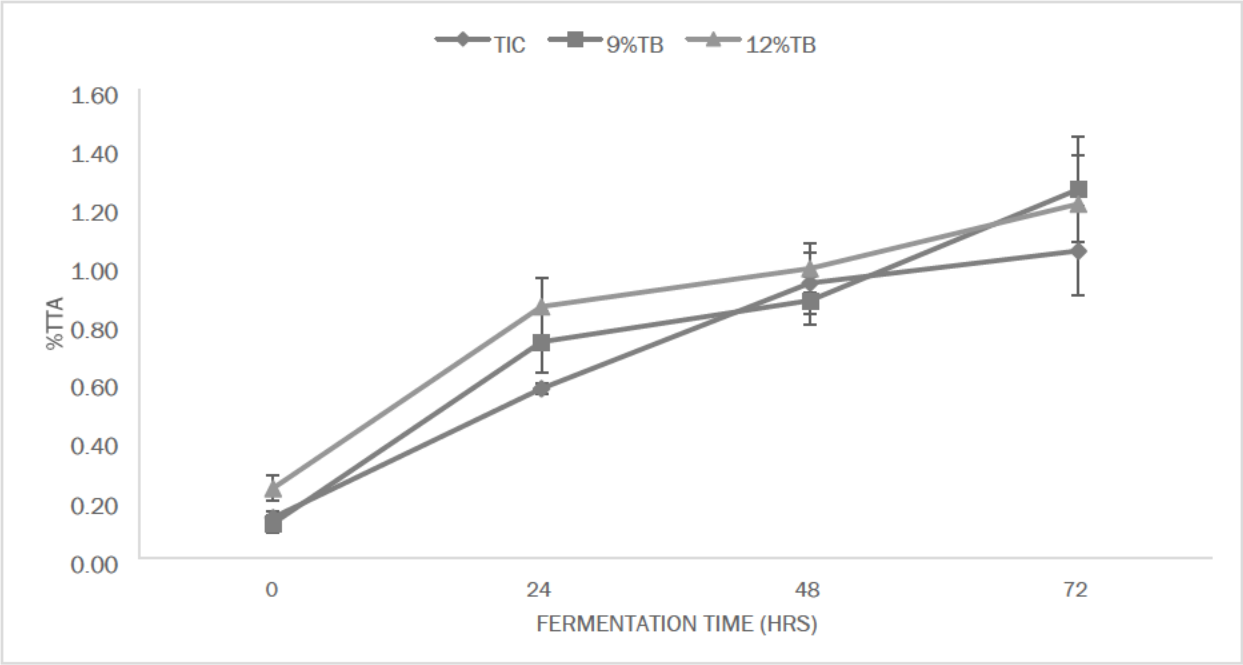

Figure 15: Effect of fermentation time on the titratable acidity of injera varieties spontaneously fermented (TIC-Tef injera; 9% BT-injera fortified with 9 % Bambara groundnut flour; 12% BT – injera fortified with 12% Bambara ground flour ($p<0.05$))

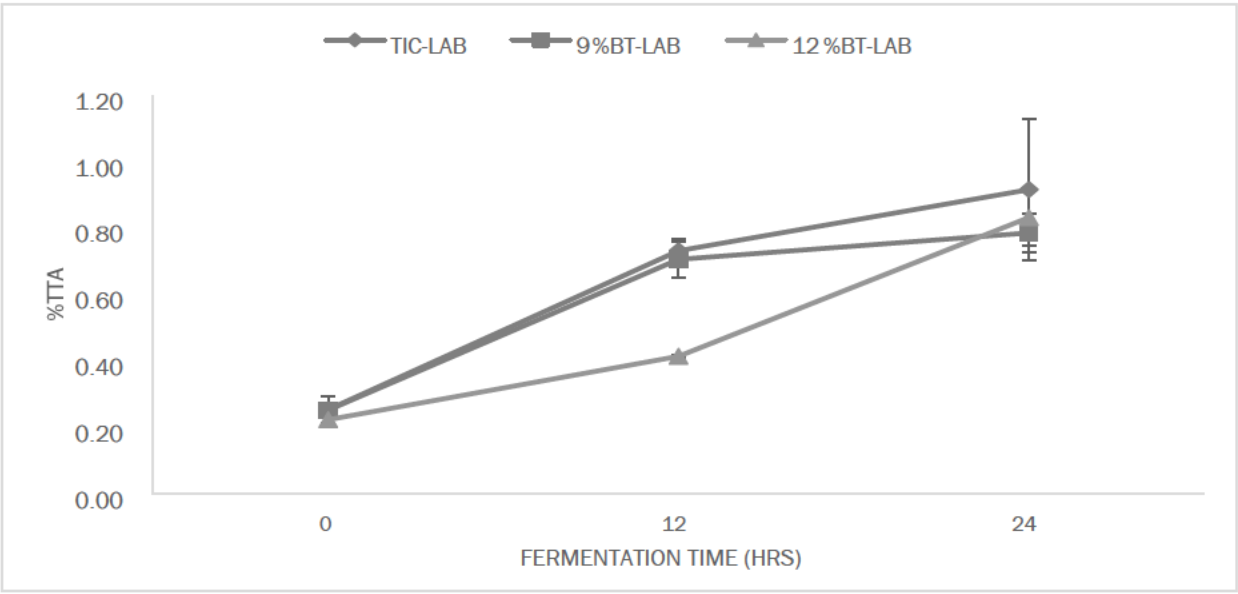

Figure 16: Effect of fermentation on the titratable acidity of injera varieties fermented using LAB starter cultures (TIC-LAB; Tef injera inoculated with LAB cultures, 9%TB-LAB; injera fortified with Bambara groundnut inoculated with LAB cultures, 12%TB-LAB; injera fortified with Bambara groundnut inoculated with LAB cultures.

4.4 CONCLUSION

The addition of LAB starter cultures (*Lactococcus lactis, lactobacillus curvatus, Weissella confusa*) at 1% substantially reduced the fermentation time. A pH below 4 was reached within 24 hours. This may be appropriate for commercial production of injera, whether supplemented with Bambara groundnut flour or originally made from Tef flour only. The use of LAB starter cultures to produce Tef injera and Bambara fortified injera may constitute to one major step towards improved safety and sensory quality of traditional fermentation. These findings show that Tef injera and Bambara fortified injera can be successfully produced commercially and locally using LAB cultures.

Chapter 5:

Nutritional quality of tef-injera and tef-injera fortified with Bambara groundnuts produced using lab starter cultures

Abstract

This study was aimed at improving the nutritional quality of tef injera by fortifying with Bambara groundnut flour and fermented using lactic acid bacteria starter cultures. The nutritional content of Tef injera and injera fortified with Bambara flour at 9 %, and 12 % was determined. Injera samples were naturally fermented and inoculated with LAB cultures isolated from injera fermentation (*Lactobacillus curvatus, Lactococcus lactis and Weissella confusa*). One-way analysis of variance was performed on the results and the means compared using Tukey post hoc test at $p<0.05$, injera fortified with 12% of Bambara groundnut and inoculated with LAB cultures (12 % TB-LAB) had a significantly high ($p<0.05$) protein of 23.21 %, the lowest protein content was recorded for Tef injera (TIC) at 7.35 %. The protein digestibility of Tef injera increased with the addition of Bambara groundnut and LAB starter culture, the digestibility of protein increased from 40 % for TIC to 80 % for injera fortified with 12 % TB-LAB. There was not a significant increase ($p >0.05$) in the amino acid content after addition with Bambara groundnut flour and LAB cultures, the amino acid concentrations were slightly lower than the standard concentration recommended by the FAO/WHO for adults. These results indicate that injera fortified with 12% of Bambara groundnut and inoculated with LAB cultures has the potential to make a significant contribution to the alleviation of malnutrition.

Keywords: Amino Acid, Lactic acid bacteria, starter cultures, protein digestibility

5.1 INTRODUCTION

Cereals are undeniable the most consumed food sources among humans, contributing more than 60% to the world's food production and are highly processed to a digestible state through the fermentation process (Waters *et al.,* 2015). Most African fermented foods consumed daily from beverages to gruels are made from subjecting the cereal crops to water, allowing them to ferment to a digestible state (Galati *et al.,* 2014). Tef (*Eragrostis tef*) is a cereal crop that is famously cultivated and utilised in Ethiopia. It is mainly used to make an Ethiopian pancake-like bread that is consumed with different stews. Tef is a gluten-free cereal crop and contains rather high Iron, Zinc and Calcium content (Girma *et al.,* 2013; Kaleab 2014). With the number of undernourished people in the world increasing in 2016 from an estimated 777 million in 2015 to 815 million in 2016, there is more work and research that needs to be carried out to reduce this number consistently (McGuire 2015)

Anti-nutrients such as phytate, typsin, proteases and polyphenols are naturally present in cereals and inhibit the digestion and absorption of micronutrients and macronutrients by the human body (Ref). According to Holzapfel (2002), fermentation decreases the activity of proteinase inhibitors. It has been shown that fermentation reduces the amount of antinutritional factors in cereal leading to increased bioavailability of nutrients and micronutrients such as Fe, Zn, Ca and Mg to the body (Chelule *et al.,* 2010)

Fermentation was observed to decrease the anti-nutritional factors and also improving mineral availability and protein digestibility of sorghum injera (Mohammed *et al.,* 2011). Khetarpaul and Chauhan (1990) reported improved mineral availability of pearl millets fermented with pure cultures of *Lactobacillus* sps and yeast. The action of Lactic acid bacteria in fermented cereal blends showed an increase in riboflavin, niacin and lysin (Charalampopoulos *et al.,* 2002).

Fermentation by addition of starter culture can improve the nutritional quality, but additional measures such as fortification can also improve the nutritional quality of the food. Fortification of already existing and popular foods with inexpensive protein sources is a feasible means of alleviating the problem of undernourishment and protein malnutrition (Ref). Under-utilised legumes such as Bambara groundnut have great potential as a fortifying agent as they are an excellent source of proteins and essential amino acids. Bambara groundnuts are African legumes, with high protein ranging between 18-24% and lysine content ranging between 6.5-6.8% and methionine of 1.8-2.84 % (Abdualrahman *et al.,* 2012; Abdualrahman *et al.,* 2019). The objective of this study is, therefore, to assess the nutritional quality content of traditional tef-injera compared to fortified Tef-injera with or without the addition of a starter culture.

5.2 MATERIALS AND METHODS

5.2.1 PREPARATION OF FLOURS

Bambara flour and tef flour were prepared as described in chapter 3 and 4.

5.2.3 FERMENTATION OF TEF-INJERA AND INJERA FORTIFIED WITH 9% AND 12% BAMBARA FLOUR

Tef-Injera and injera fortified with 9% and 12% Bambara flour was prepared according to the methods described by Mezemir (2015) and Boka *et al.* (2013) in 900 ml batches. Tef flour (300 g) and filtered water (600 ml) were mixed together in sterile 2L Erlenmeyer flasks and incubated at 25±2˚C for 72 hours. After 30 hours of fermentation, a yellow liquid accumulating on the batter was discarded and replaced with filtered water and left to further ferment for 42 hours. About 10 % of the fermenting dough was mixed with three parts of water and boiled for 2-5 minutes to form "absit". The absit was cooled to 60˚C and transferred back to the remaining fermented batter and allowed to ferment for two hours (secondary fermentation). After two hours the batter was baked

on a mitad for 2 minutes at 220˚C. Injera fortified with Bambara flour with 9 % of Bambara flour was prepared by weighing out 27 g of Bambara flour and 273 g of tef flour with 600 ml of filtered water to ferment. Injera fortified with 12% Bambara flour, was prepared by adding 36 g of Bambara flour and 264 g of Tef flour and mixed with 600 ml of filtered water to ferment. Injera samples inoculated with LAB starter cultures were fermented as previously described in chapter 4.

5.2.4 DETERMINATION OF ASH CONTENT

Crucibles were first dried and cooled onto a desiccator, weighed accurately and mass recorded according to the AOAC Official Methods (AOAC 2000) with few modifications. About 3g of the sample was weighed on a crucible and burnt in the muffle furnace at 600 ˚C for 6 hours until all organic material has volatilized. The ashed crucible was cooled in a desiccator. After cooling, it was weighed, and the mass of residue was determined and expressed as a percentage in the whole sample.

$$\%\ Ash = \frac{mass\ of\ ash(g)}{mass\ of\ sample\ (g)} \times \frac{100}{1}$$

5.2.5 MOISTURE CONTENT DETERMINATION

The moisture content was determined according to the AOAC Official Methods (AOAC 2000). A dried and cooled crucible was weighed, and its mass recorded. Exactly 3g of the sample was weighed onto the crucible and placed in a drying oven at 105˚C for 3 hours. The crucible was removed and allowed to cool in a desiccator for 20 minutes. After cooling the mass of residue determined.

$$\%\ Moisture\ = \frac{Sw1 - Sw2}{Sw1} \times \frac{100}{1}$$

Where,

SW1- weight (g) of sample before drying

SW2- weight (g) of sample after drying

5.2.6 CRUDE PROTEIN USING BUCHI 430 DIGESTER AND BUCHI 321 DISTILLATION UNIT

Crude protein was determined using the Kjeldahl method as described in (AOAC 2000). About 3 g of the sample was weighed into digestion tubes, and 4 g of catalyst mixture and 25 ml of concentrated H_2SO_4 (98%). Escape of noxious fumes was avoided by connecting the digestion tubes to a NaOH trap. All unused openings were closed with a cotton plug. Heating was initiated and maintained such that the sample was always boiling. Digestion proceeded for approximately1-2 hours and completed when the solution has turned light clear green.

The digested sample was diluted in an approximate ratio of 1:3. A 250 ml Erlenmeyer receiving flask was prepared by adding 25 ml of 2 % boric acid and six drops of screened methyl red indicator. The Erlenmeyer receiving flask was placed under long tubes and 32 % of sodium hydroxide solution was added by pressing on the NaOH switch until it turned to a dark brown colour. Distillation time was set to 2 % for three minutes, and the distillate was then titrated with a standard 0.1 N Sulphuric acid. The end point was reached when the light blue solution turned colourless to grey.

$$\% N = \frac{Titration\ in\ ml - Blank\ in\ ml)\ \times 1.4 \times 0.1}{Mass\ of\ Sample}$$

$$\% Protein\ = factor\ (for\ product) \times N$$

5.2.7 DETERMINATION OF FAT CONTENT

About 3 g of sample was weighed on a crucible and placed into an incubator at 105˚C overnight with the bottom flask stable as described in AOAC (2000). The sample was extracted for fat using 200 ml of petroleum ether and filtered using a filter paper on a bottom flask. The bottom flask was connected on to the soxhlet apparatus, and water turned to cool them, and the heating mantle turned on. The sample could be heated for two runs after condensation before turning the heating mantle off. The bottom flask was then cooled for 3-4 hours before being reweighed for their dry content.

$$\% Fat = \frac{weight\ of\ fat}{weight\ of\ sample} \times \frac{100}{1}$$

5.2.8 TOTAL CARBOHYDRATES

Total carbohydrates (%) was determined by difference according to the AOAC Official Methods with the exclusion of crude fibre.

Carbohydrate % = (protein + fat + Moisture + Ash - 100)

5.2.9 IN-VITRO PROTEIN DIGESTIBILITY

According to the method of Ayo *et al.* (2007), about 200 mg of each injera samples were weighed into Erlenmeyer flasks and mixed with 1.5 mg of pepsin in 0.1M KH_2PH_4, pH 2.00. Samples were

digested for 24 hours at 37 ˚C in a shaking water bath. Digestion was stopped by adding 2 ml of 2 M NaOH. Samples were centrifuged (4,900xg, 40˚C) for 20 minutes, and the supernatant discarded. The residues were washed and centrifuged twice with 20 ml buffer (0.1M KH_4PO_2, P^H 7.0). Undigested nitrogen was determined using the Kjeldahl method. Digestibility was calculated as;

$$\%\ Digestibility = \frac{N\ in\ sample - Undigestible\ N}{N\ in\ Sample} \times \frac{1}{100}$$

5.2.10 DETERMINATION OF AMINO ACID

Amino acids content was determined using the method described by Grobbelaar *et al.* (2014) with slight modification. Amino acid separation and detection were performed using a Waters Acquity Ultra Performance liquid Chromatography (UPLC) fitted with a photodiode array (PDA) detector. Derivatisation kit, according to the manufacturer's guide. Sample /standard solution (1ul) was injected into the mobile phase (AccQ-Tag Ultra Eluent A and B (Waters)), which conveys the derivative amino acid onto a Waters Ultra Tag C_{18} column (2.1 x 50 mm x 1.7 um) held at 60˚C.

5.2.11 STATISTICAL ANALYSIS

Samples for proximate analysis were analysed in triplicate, and each analysis was repeated at least in triplicate. Mean and standard deviations were calculated, and one-way analysis of variance (ANOVA) was done. Mean separation was done by Tukey post hoc for significant difference amongst means ($p<0.05$), using IBM SPSS Statistics software

5.3 RESULTS AND DISCUSSION

5.3.1 PROXIMATE COMPOSITION OF FLOURS

The nutritional composition of flours differs depending on the origin, type, and processing; therefore, it is important to know the nutritional quality of raw materials. This will assist with the reasoning and understanding of the nutritional quality of the finished product. The protein content of Bambara groundnut flour which is presented in Table 1, 19.52% was significantly higher ($p < 0.05$) than the protein content of Tef flour 9.98%. Gebremariam *et al.,* (2014a) and Bultosa (2007), reported protein content range between 8.7-11.1% with a mean of 10.4% for 13 Tef varieties. Legumes are generally richer in protein compared to cereal grains (Temba *et al.,* 2016). Bambara flour also had significantly higher ($p < 0.05$) crude fat content 7.04% compared to Tef flour 2.50% (Table 4) with highest carbohydrate content observed in Tef flour (75.10%) than Bambara flour (61.66%). Bambara groundnut flour's crude protein, ash, crude fat, moisture, carbohydrate and energy (19.52%, 2.13%, 7.04%, 9.70%, 61.66% and 375Kj) findings were similar to what has been reported by Abdualrahman *et al.* (2012) and Adeleke *et al.* (2018).

5.3.2 PROXIMATE COMPOSITION OF INJERA PRODUCTS

The protein content of injera made from Tef flour only, as it is natively produced, was analysed for its proximate composition. An increase in protein content was observed with injera fortified with Bambara groundnut flour, and additionally, an increase was observed with the inoculation of LAB cultures. The protein content for all the samples increased in the following order TIC < 9%TF < 12%TB < TIC-LAB < 9% TB-LAB < 12%TB-LAB. Tef injera fortified with 12% Bambara groundnut, and fermented using LAB cultures had a significantly higher ($p < 0.05$) protein content when compared to all other injera products (Table 4). Bambara groundnut and cooked banana, had been added to fermented maize dough increasing the protein content of these weaning foods (Mbata *et al.,* 2009; Abdualrahman *et al.,* 2012). Some LAB species have the potential to over

produce, and release important nutrients during fermentation (Ogunremi *et al.,* 2017). Anti-nutrients from cereals and legumes can be reduced by fermenting with LAB starter cultures which produce low molecular weight organic acid at a faster rate, lowering the pH which is beneficial for phytase activity (Moroni *et al.,* 2015).

A significant increase ($p<0.05$) was also observed in terms of ash content. Tef injera had a lower ash content of 0.89% increasing to 1.52 % observed for the injera sample supplemented with 12% Bambara flour, and fermented using LAB cultures, as shown in Table 4. The fat content of injera was the highest for tef-injera, as shown in Table 1 (1.81%), and decreased with the addition of Bambara groundnut flour and after fermentation with LAB cultures.

The moisture content of tef-injera 67.53% was significantly different ($P<0.05$) to the moisture content of injera product supplemented with 9% and 12% Bambara groundnut with 59.77 % and 59.48 % respectively, as shown in Table 4. The moisture content of injera ranges between 63-65 % (Ashagrie and Abate 2012). The moisture content of injera is important because it affects its texture. Injera has to roll without breaking or sticking. and has to be able to grab and soak up stews (Zegeye 1997; Attuquayefio 2014). Injera produced with tef flour only had the highest carbohydrate content, and the injera samples supplemented with Bambara groundnut flour, and inoculated with LAB cultures had the lowest carbohydrate content (Table 4) this may be due to carbohydrates being utilised by microorganisms as a source of energy to produce carbon dioxide and possibly liberate other nutrients. The gross energy of tef-injera was significantly higher ($p < 0.05$) when compared to injera produced by supplementing with Bambara groundnut flour and those also inoculated with LAB cultures as presented in Table 4.

Table 4: Proximate composition of Tef flour, Bambara flour and injera products (g/100g)

Samples	Moisture	Ash	Fat	Protein	Carbohydrates	Energy (Kj)
TF	9.88 ± 0.34^{e}	2.52 ± 0.60^{d}	2.50 ± 0.48^{e}	9.98 ± 1.52^{e}	75.10 ± 1.89^{e}	362.85 ± 2.55^{e}
BF	9.70 ± 0.60^{f}	2.13 ± 0.01^{d}	7.04 ± 0.23^{d}	19.52 ± 0.20^{f}	61.66 ± 0.50^{f}	387.46 ± 3.46^{f}
TIC	67.53 ± 1.22^{a}	0.89 ± 0.05^{a}	1.81 ± 0.18^{a}	7. 35 ± 0.29^{a}	22.32 ± 1.16^{a}	134.97 ± 5.28^{a}
9 % TF	59.77 ± 0.86^{b}	1. 39 ± 0.12^{b}	1.23 ± 0.05^{b}	16.87 ± 0.06^{b}	20.74 ± 0.87^{a}	160.51 ± 2.95^{b}
12% TF	59.48 ± 0.51^{b}	1.45 ± 0.26^{c}	1.67 ± 0.86^{b}	16.86 ± 0.45^{b}	12.18 ± 0.43^{b}	164.20 ± 2.49^{b}
TIC-LAB	67.13 ± 0.23^{a}	1.48 ± 0.06^{c}	0.88 ± 0.23^{c}	22.1 2± 0.09^{c}	8.38 ± 0.21^{c}	129.96 ± 1.98^{a}
9% TB-LAB	64.37 ± 0.31^{d}	1.25 ± 0.01^{c}	1.52 ± 0.08^{b}	22.33 ± 0.05^{c}	10.51 ± 0.22^{d}	142.68 ± 0.92^{d}
12% TB-LAB	63.27 ± 0.27^{d}	1.52 ± 0.18^{c}	1.63 ± 0.11^{b}	23.21 ± 0.23^{d}	10.17 ± 0.53^{c}	147.20 ± 2.08^{d}

Mean (n=3) is reported. Mean with different superscript letters in column are significantly different ($p>0.05$)

5.3.3 IN-VITRO PROTEIN DIGESTIBILITY OF INJERA

Injera fortified with 12 % injera and fermented by inoculating LAB cultures (12 %TB-LAB) had the highest protein digestibility (approx. 80%) as depicted in Figure 17. Protein digestibility also increased with the addition of Bambara flour (Figure 17). Awobusuyi (2016) also reported similar findings with an increase in the digestibility of Amahewu with the inclusion of Bambara flour. The in-vitro protein digestibility of Tef flour has been reported to range between 71-72 % which is relatively higher than other gluten-free cereals (Antony and Chandra, 1998). Shumoy and Raes (2015) and Shumoy *et al.* (2018) reported an increase in IVPD on tef injera samples after fermentation. The effect of fermentation on the digestibility of protein content has been studied in different legumes and cereals. A partial or complete elimination of tannins, phytic acid and trypsin inhibitor activity was reported for legumes as the consequence of lactic acid bacteria fermentation (Coda *et al.,* 2010). During fermentation, microorganisms produce proteolytic enzymes which may be responsible for the increased protein digestibility. Monawar (1983) and Fischer *et al.* (2014) found that the reduction in pH during fermentation also enhances the activity of native proteolytic enzymes, and consequently promotes the breakdown of proteins to smaller polypeptides which are easily digested by enzymes. Overall, an improvement in digestibility will lead to better protein absorption, and retention in humans following the consumption of protein fortified injera (Urga *et al.,* 2017)

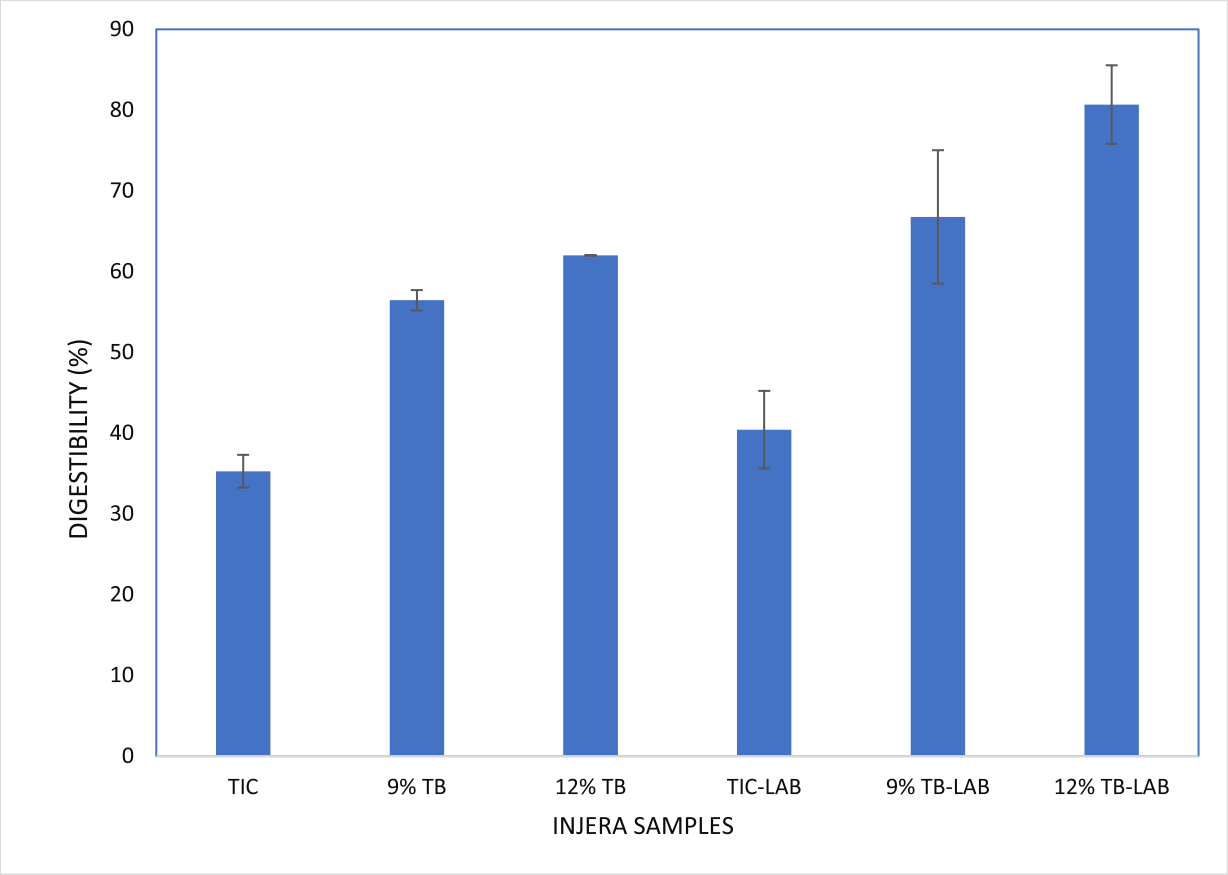

Figure 17: In-vitro protein digestibility of different injera products, TIC= Tef injera, 9%TB= Tef injera fortified with 9% Bambara groundnut flour, 12%TB = Tef injera fortified with 12% Bambara groundnut flour, TIC-LAB = Tef injera + LAB cultures, 9% TB-LAB = Tef injera fortified with 9% Bambara flour + LAB cultures, 12%TB-LAB = Tef injera fortified with Bambara flour + LAB cultures.

5.3.4 AMINO ACID

Table 5 shows the amino acid composition consisting of essential and non-essential amino acids for injera samples that have been fortified with Bambara groundnut flour and fermented with LAB cultures. Among all the amino acid analysed, glutamic acid was highest in all samples followed by leucine, histidine and lysine. The amino acid amount in all samples was slightly lower than the amounts recommended by the FAO/WHO standard concentration (FAO 1985). Supplementing the injera with high protein legume, and fermenting the flours by inoculating with LAB cultures did

not have a high impact on the amino acid composition of the proteins in the samples analysed. Awobusuyi (2016) showed that there was an increase in amino acid of Amahewu after the addition of Bambara groundnut, and inoculation with a starter culture. A similar trend was observed with the fermentation of sorghum injera (Mohammed *et al.,* 2011).

The amounts of all the amino acid analysed varied in the following order: glutamic acid > Leucine >Phenylalanine > Aspartic acid > Alanine > Valine > Serine > Proline > Tyrosine > Threonine > Arginine > Methionine > Isoleucine > Glycine > Lysine > Histidine. The amounts of essential amino acid however ranged in the following order: Leucine >. Phenylalanine > Threonine > Valine > Methionine > Isoleucine >lysine >Histidine. In cereal fermentations enzymes determine proteolysis, peptidase activity of sourdough LAB contributes significantly to the hydrolysis of peptides, The presence of Gly, Ala, Val, Leu, Tyr and Phe peptides all impart bitterness (Zhao *et al.,* 2015). Glutamate, which was highest in all injera products, is said to impart an umami taste in cereal fermented foods.

Table 5: Amino acid composition of injera and injera fortified with Bambara groundnuts flour (g/100g protein)

AMINO ACID	TIC	9 % TF	12 % TF	TIC-LAB	9% TB-LAB	12% TB-LAB	FAO/WHO recommended pattern for Adult
***His**	0.23	0.28	0.3	0.34	0.40	0.35	**1.6**
Ser	0.44	0.55	0.56	0.60	0.66	0.67	
Arg	0.29	0.4	0.45	0.44	0.55	0.53	
Gly	0.42	0.51	0.52	0.41	0.48	0.47	
Asp	0.45	0.57	0.69	0.61	0.80	0.85	
Glu	2.15	2.24	2.49	2.74	2.87	3.26	
***Thr**	0.34	0.34	0.4	0.54	0.54	0.56	**0.9**
Ala	0.52	0.56	0.55	0.35	0.62	0.72	
Pro	0.59	0.72	0.68	0.54	0.57	0.62	
***Lys**	0.15	0.16	0.2	0.35	0.33	0.37	**1.6**
Tyr	0.4	0.57	0.57	0.74	0.66	0.61	
Met	0.36	0.42	0.35	0.65	0.52	0.52	**1.7**
Val	0.53	0.58	0.6	0.59	0.65	0.69	**1.3**
Ile	0.48	0.64	0.78	0.40	0.50	0.49	**1.3**
Leu	0.83	0.98	0.98	0.94	1.04	1.06	**1.9**
Phe	0.65	0.82	0.84	0.99	1.07	0.86	**1.9**

***Essential Amino acid**

5.4 CONCLUSION

Injera supplemented with 12 % Bambara groundnut and LAB cultures has a better nutritional composition in terms of protein content, and protein digestibility. There was a slight increase of amino acid after the addition of Bambara flour and fermentation with LAB cultures. Injera fortified with Bambara groundnut has the potential in making a significant contribution towards alleviating malnutrition.

Chapter 6:

Storage stability of tef injera fortified with Bambara flour, fermented using lactic acid bacteria.

Abstract

In this study, the storage stability of tef injera and injera fortified with Bambara groundnut was determined. Tef injera samples and injera fortified with Bambara groundnut samples were fermented spontaneously, using LAB starter cultures. A total of six injera samples were subjected to different storage conditions at 4˚C and 25˚C and monitored for three days. Injera samples were observed and plated every day for three days to assess the presence of aerobic spore-formers, moulds, coliforms and total microbial load. There was a significant difference ($p < 0.05$) on the microbial quality of injera samples stored at refrigeration temperature compared to samples stored at room temperature from day 1 to day 3. There were no microbial counts at 6˚C for all samples from day 0 to day 3, except for Tef injera and Tef injera inoculated with LAB cultures, which were observed to have a total plate count of 4.68 log (CFU/g) and 4.42 log (CFU/g). Tef injera also had growth on the day 3 at 25˚C with coliform, mould and total plate growth, but for Tef injera inoculated with LAB cultures, there was no growth. Throughout the storage period in both conditions, aerobic spore formers were not isolated. Refrigeration temperatures effective in preservation technique, maintained the microbiological quality of injera for three days.

Keywords: Microbiological quality, Preservation, storage stability

6.1 INTRODUCTION

Injera is consumed to a great extent in Ethiopia. There is a developing interest in the product and its grain not only because of its gluten status but also its nutrients. Injera can be made not only from tef but also from rice flour and sorghum to mention a few (Assefa *et al.,* 2018). Once injera has been baked, it is normally stored at room temperature, although the storage period does not usually exceed three days at ambient temperatures (Ashagrie and Abate 2012). Injera has a high moisture content when compared to other flat breads or breads. The moisture content of injera which ranges between 62-65 %, make injera more susceptible to mould spoilage (Attuquayefio 2014). Contamination of baked goods normally occurs after baking. Airborne distribution of mould spores and dust causes most spoilage (Chavan and Chavan 2011). Mould can be dangerous in the sense that they may produce toxic metabolites known as mycotoxins, and their control is difficult (Dalié *et al.,* 2010).

Numerous studies have been conducted on the microbial quality of fermented foods of low and high-water content stored at refrigeration and ambient temperatures. Studies showed that at refrigeration temperatures microorganisms were not prevalent, thereby extending their shelf life (Awobusuyi 2015). Fermented foods such as ogi, kunun-zaki and togwa stored under refrigerated conditions had reduced microbial load compared with that stored at ambient temperatures (Gaffa *et al.,* 2002; Ohenhen and Ikenebomeh 2007; Dike and Sanni 2010). Chemical preservatives, and their effectiveness have been explored on their potential to improve the shelf life of injera (Ashagrie and Abate 2012; Hassen *et al.,* 2018). Therefore, the effect on the addition of Bambara groundnut flour and LAB starter cultures on the microbiological quality of injera fortified with Bambara groundnut flour in different storage condition; room (± 25˚C) and refrigeration (± 4˚C) were investigated.

6.2 MATERIALS AND METHODS

6.2.1 DETERMINATION OF AEROBIC SPORE-FORMERS

Tryptone Soy Agar was prepared, sterilised, and stored at 50 ˚C until use. Injera samples were heated in a sterile test tube, and held for 20 minutes at 75˚ C (Austin 1998). Serial dilutions were pour plated in triplicate, and the inverted set of plates were incubated aerobically at 35˚C for 48-72 hours. Three triplicates of each sample were analysed.

6.2.2 DETERMINATION OF TOTAL COLIFORMS

Injera samples were serially diluted using MacConkey agar, and inoculated into petri dishes. The plate was incubated for 24-48hours; after which colonies were then counted, and expressed as log cfu/ml (Chacko *et al.,* 2010). Three replicates of each sample were analysed.

6.2.3 DETERMINATION OF YEAST AND MOULDS

Determination of the presence of yeast and moulds was done according to the method of Beuchat (1992), and modified. Potato dextrose agar (PDA) was prepared onto petri dishes, and injera samples were serially diluted into media. The petri dishes were incubated at room temperature for 48-72 hours. Three replicates of each sample were analysed.

6.2.4 DETERMINATION OF TOTAL PLATE COUNT

Injera samples were serially diluted and inoculated into petri dishes using plate count agar. The plate was incubated for 24-48hours, colonies were then counted, and expressed as log cfu/ml. Three replicates of each sample were analysed.

6.2.5 DETERMINATION OF LACTIC ACID BACTERIA

DeMann Rogosa-shape (MRS) was prepared and cooled to 45˚C. The agar was poured into a petri dish where appropriate serial dilution was pipetted onto plates in triplicates. The plates were inverted and incubated at 37˚C for 48 hours anaerobically. Three replicates of each sample were analysed.

6.2 RESULTS AND DISCUSSION

The stability of injera samples varied with storage time and period, no growth was observed for all injera samples stored at 4˚C and 25˚C from day 0-1 (Tables 6,7,8 and 9). This could be due to the baking conditions of injera samples. Injera is prepared by spreading the batter in the pan ("mitad") and baking for 2-3 minutes at 230˚C, which could result in the destruction of microorganisms. According to Ashenafi (2006), the temperature in the middle of the injera during the baking process could reach around 90˚C. Microbial growth was observed during the second day of storage at 25˚C. The production of lactic acid reduced the pH; therefore, at the final stages of the fermentation, the pathogenic bacteria were reported not to have survived in the fermented food as presented in Figure 13 and 16 (Chapter 2) (Sahlin and Nair 2012).

Tef-injera (TIC) was isolated for total microbial, coliforms, yeast and mould, and this could be because of tef injera's moisture content (65%), there was a reduction in the moisture content due to the addition of Bambara groundnut and the use of LAB starter culture. As a result, it had an impact on its storage stability of the injera and injera fortified with Bambara flour. Coliform was not observed for other samples stored at room temperature and refrigeration temperature. Aerobic spore-formers were also not present from day 0-3 (table 6-8) for all injera samples.

On the third day at 25˚C it was observed that the following, TIC had the highest coliform growth (6.16 log (CFU/g), the highest yeast and mould (7.54 log (CFU/g) count was observed for 9% BT

and for total plate count (9.26 log (CFU/g) was observed for 12% BT (Table 9). Microbial counts were low at 4˚C, whilst higher counts were observed at room temperature. Throughout the storage period in both conditions, aerobic spore formers were not isolated. The high bacterial population in samples could be due to the high moisture content of the bread and the presence of nutrients from the Bambara groundnut flour, providing favourable conditions for the growth of microorganisms. Ajibade (2018), investigated the microbial load of fortified bread, which is wheat-milled Bambara bread at room and refrigeration temperatures, the author found that the bread samples stored at refrigeration temperatures had significantly lower ($p<0.05$) microbial growth when compared to those stored at room temperatures. Refrigeration temperatures effectively maintain the microbiological quality of injera for three days.

Table 6: Storage stability of six different injera stored at refrigeration temperature and room temperature (day 0)

Injera products	Mould (logcfu)	Total coliform count	Aerobic spore formers	Total plate count
TIC	ND	ND	ND	ND
9% TB	ND	ND	ND	ND
12% TB	ND	ND	ND	ND
TIC-LAB	ND	ND	ND	ND
9% TB-LAB	ND	ND	ND	ND
12% TB-LAB	ND	ND	ND	ND

ND: Not Detected, TIC: Tef injera, 9% TB: Tef injera fortified with 9% Bambara flour, 12% TB: Tef injera fortified with 12% Bambara flour, TIC-LAB: Tef injera with LAB cultures, 9%TB-LAB: Tef injera fortified with 9% Bambara flour with LAB cultures, 12% TB-LAB: Tef injera fortified with 12% Bambara flour with LAB cultures. Mean with different superscript letters in the same column are significantly different ($p<0.05$) according to the Tukey test.

Table 7: Storage stability of six different injera stored at refrigeration temperature and room temperature (day 1)

DAY 1 - 4˚C				
Injera products	**Mould (logcfu)**	**Total coliform count**	**Aerobic spore formers**	**Total plate count**
TIC	ND	ND	ND	ND
9% TB	ND	ND	ND	ND
12% TB	ND	ND	ND	ND
TIC-LAB	ND	ND	ND	ND
9% TB-LAB	ND	ND	ND	ND
12% TB-LAB	ND	ND	ND	ND
25˚C				
Injera products	**Mould (logcfu)**	**Total coliform count**	**Aerobic spore formers**	**Total plate count**
TIC	ND	ND	ND	ND
9% TB	ND	ND	ND	ND
12% TB	ND	ND	ND	ND
TIC-LAB	ND	ND	ND	ND
9% TB-LAB	ND	ND	ND	ND
12% TB-LAB	ND	ND	ND	ND

ND: Not Detected, TIC: Tef injera, 9% TB: Tef injera fortified with 9% Bambara flour, 12% TB: Tef injera fortified with 12% Bambara flour, TIC-LAB: Tef injera with LAB cultures, 9%TB-LAB: Tef injera fortified with 9% Bambara flour with LAB cultures, 12% TB-LAB: Tef injera fortified with 12% Bambara flour with LAB cultures. Mean with different superscript letters in the same column are significantly different ($p<0.05$) according to the Tukey test.

Table 8: Storage stability of six different injera stored at refrigeration temperature and room temperature (day 2)

DAY 2 - 4˚C				
Injera products	**Mould (logcfu)**	**Total coliform count**	**Aerobic spore formers**	**Total plate count**
TIC	ND	ND	ND	ND
9% TB	ND	ND	ND	ND
12% TB	ND	ND	ND	ND
TIC-LAB	ND	ND	ND	ND
9% TB-LAB	ND	ND	ND	ND
12% TB-LAB	ND	ND	ND	ND

25˚C				
Injera products	**Mould (logcfu)**	**Total coliform count**	**Aerobic spore formers**	**Total plate count**
TIC	4.60 ± 0.09^{a}	4.80 ± 0.05	ND	5.90 ± 0.04^{a}
9% TB	6.04 ± 0.01^{b}	ND	ND	6.16 ± 0.09^{b}
12% TB	4.66 ± 0.06^{a}	ND	ND	6.05 ± 0.04^{a}
TIC-LAB	ND	ND	ND	4.90 ± 0.06^{c}
9% TB-LAB	5.05 ± 0.01^{c}	ND	ND	5.16 ± 0.01^{a}
12% TB-LAB	4.87 ± 0.02^{a}	ND	ND	5.26 ± 0.08^{a}

ND: Not Detected, TIC: Tef injera, 9% TB: Tef injera fortified with 9% Bambara flour, 12% TB: Tef injera fortified with 12% Bambara flour, TIC-LAB: Tef injera with LAB cultures, 9%TB-LAB: Tef injera fortified with 9% Bambara flour with LAB cultures, 12% TB-LAB: Tef injera fortified with 12% Bambara flour with LAB cultures. Mean with different superscript letters in the same column are significantly different ($p<0.05$) according to the Tukey test

Table 9: Storage stability of six different injera stored at refrigeration temperature and room temperature (day 3).

DAY 3 - 4˚C				
Injera products	**Mould (logcfu)**	**Total coliform count**	**Aerobic spore formers**	**Total plate count**
TIC	ND	ND	ND	4.68 ± 0.04
9% TB	ND	ND	ND	ND
12% TB	ND	ND	ND	ND
TIC-LAB	ND	ND	ND	4.42 ± 0.10
9% TB-LAB	ND	ND	ND	ND
12% TB-LAB	ND	ND	ND	ND
25˚C				
Injera products	**Mould (logcfu)**	**Total coliform count**	**Aerobic spore formers**	**Total plate count**
TIC	6.09 ± 0.01^{a}	6.16 ±0.02	ND	7.40 ± 0.04^{a}
9% TB	7.54 ± 0.06^{b}	ND	ND	7.38 ± 0.02^{a}
12% TB	6.25 ± 0.02^{a}	ND	ND	9.26 ± 0.02^{b}
TIC-LAB	5.37 ± 0.02^{c}	ND	ND	6.15 ± 0.12^{c}
9% TB-LAB	7.54 ± 0.13^{b}	ND	ND	7.51 ± 0.03^{d}
12% TB-LAB	6.56 ± 0.01^{a}	ND	ND	7.18 ± 0.01^{a}

ND: Not Detected, TIC: Tef injera, 9% TB: Tef injera fortified with 9% Bambara flour, 12% TB: Tef injera fortified with 12% Bambara flour, TIC-LAB: Tef injera with LAB cultures, 9%TB-LAB: Tef injera fortified with 9% Bambara flour with LAB cultures, 12% TB-LAB: Tef injera fortified with 12% Bambara flour with LAB cultures. Mean with different superscript letters in the same column are significantly different ($p<0.05$) according to the Tukey test.

6.3 CONCLUSION

Tef injera and injera fortified with Bambara were stable for at least three days, samples fermented by inoculating with LAB starter cultures had lower microbial spoilage and were stable for more than three days at cold storage. However, the presence of coliforms on Tef injera was observed on the second day when stored at 25˚C. Moulds were also observed on almost all the samples on the second day; this can be worrying to injera consumers who do not have access to refrigerators. Cold storage was found most suitable for the storage of Injera.

Chapter 7:

7.1 GENERAL DISCUSSION

Injera produced by supplementing with Bambara groundnut flour at 9 % and 12 % showed an improvement in the nutritional quality of the finished product. The addition of lactic acid bacteria starter cultures for the fermentation of injera and injera fortified with Bambara groundnut also proved to increase the proximate composition, in-vitro-digestibility and amino acid content of the final product when compared to injera products fermented naturally. The addition of legumes or their flours to the production of cereal fermented staple foods has the potential to decrease the problem of protein-energy malnutrition steadily.

The first part of the study was carried out to identify and characterise the lactic acid bacteria (LAB) and yeast involved in the spontaneous fermentation of traditional tef-injera and the newly developed injera like-product, which contains 12 % Bambara groundnuts at different fermentation intervals of 0, 24, 48, and 72 hour. A total of 70 LAB isolates and 30 yeast isolates were identified from both fermentations using rep-PCR fingerprinting followed by sequencing the 16S rRNA gene and the D1/D2 region of the 26S rRNA gene. *Weissella confusa Lactococcus lactis* and *Lactobacillus curvatus* were the predominating microorganisms for the fermentation of tef injera and injera fortified with Bambara groundnut flour. Enterobacteriaceae predominated at the beginning of the spontaneous fermentation of injera fortified with 12 % Bambara but later in the fermentation Lactococcus *lactis* and *Weissella confusa* dominated from 24-72 hours Yeast isolates identified in both fermentations were non-fermenting yeast with *Cryptococcus* being the dominating yeast species. The unambiguous identification of LAB and yeast from tef-injera and injera fortified with Bambara groundnut using molecular biology-based methods is the first step in the future development of defined starter cultures as a feasible means of optimising the fermentation of injera.

The fourth part of the study evaluated the impact of isolated lactic acid bacteria from the first study on the pH and titratable acidity of tef injera and injera fortified with Bambara groundnut at 9 % and 12 %. The isolate combination (*Lactococcus lactis, Weissella confuse* and *Lactobacillus curvatus*) showed an ability to ferment as judged by lowering the pH of Tef injera; injera fortified with 9% Bambara flour and 12 % from 5.73 to 3.78, 5.63 to 3.80 and 5.60 to 3.81 respectively within 24 hours of the fermentation than when compared to spontaneously fermented Tef injera batter and injera fortified with Bambara flour. A similar trend was also observed with an increase in titratable acidity of the Tef injera and fortified injera samples from 0.26 to 0.92%, 0.25 to 0.79 % and 0.22 to 0.83 % respectively. Naturally, fermented injera and injera fortified samples showed a slower reduction of pH to below 4.00 and a slower increase in titratable acidity after 72 hours.

The fifth part of the study investigated the influence of fortifying injera and the addition of a starter culture on the proximate composition, in-vitro protein digestibility and amino acid content of the final products. Injera fortified with 12 % Bambara groundnut with LAB culture had a significantly high ($p < 0.05$) protein of 23.21 %, the lowest protein content was recorded for Tef injera at 7.35%. The protein digestibility of Tef injera increased with the addition of Bambara groundnut and LAB starter culture. The digestibility of protein increased from 40 % for Tef injera to 80 % for injera fortified with 12% Bambara flour with LAB culture. There was not a significant increase ($p > 0.05$) in the amino acid content after addition with Bambara flour with LAB cultures. The amino acid concentrations were slightly lower than the standard concentration recommended by the FAO/WHO for adults. These results indicate that injera fortified with 12 % of Bambara groundnut with LAB cultures has the potential to make a significant contribution to the alleviation of malnutrition. The sixth

part of the study showed the influence of two different storage temperatures on the microbial stability of tef injera and injera fortified with Bambara groundnut.

A total of six injera samples were subjected to different storage conditions: 4˚C and 25˚C There were no microbial counts at 4˚C for all samples from day 0 to day 3, except for Tef injera and Tef injera with LAB cultures, which were observed to have a total plate count of 4.68 log (CFU/g) and 4.42 log (CFU/g). On the third day at 25˚C tef-injera control had the highest coliform growth (6.16 log (CFU/g), the highest yeast and mould (7.54 log (CFU/g) count was observed for 9% injera fortified with Bambara groundnut and for total plate count (9.26 log (CFU/g) was observed for 12% injera fortified with Bambara groundnut (table 9). Microbial counts were low at 4˚C, whilst higher counts were observed at room temperature. Throughout the storage period in both conditions, aerobic spore formers were not isolated. Refrigeration temperatures effectively maintain the microbiological quality of injera for three days.

This research showed that fortification by means of substituting legume flours to the formulation of cereal fermented foods could improve the nutritional quality of the final product, the addition of LAB starter cultures also elevated the nutritional quality of injera and injera fortified with Bambara groundnut flour. The newly formulated injera, which was fortified with Bambara groundnut had a stable shelf life at refrigeration temperature for three days.

8. CONCLUSION AND RECOMMENDATIONS

The objectives for this study were achieved, lactic acid bacteria species which were predominating during the fermentation of injera and Bambara groundnut identified and isolated for use for the control fermentation. The lactic bacteria starter culture mixture had the capacity of increasing the titratable acidity and decreasing the pH of the fermented better within 24 hours, improving the fermentation period of the process from 72 hours to 24 hours. A significant improvement in the nutritional quality and storage stability was also observed for tef injera and injera fortified with Bambara groundnut flour inoculated with LAB starter cultures. The inclusion of legumes in the production of cereal fermented staple foods is a feasible measure, especially for developing countries for improving the nutritional quality of foods. The addition of Bambara groundnut proved to increase the protein content, the ash and digestibility of the available proteins. The use of LAB starter cultures isolated from the injera fermentation also proved to optimise the fermentation allowing for a reduced fermentation period and improved overall proximate composition.

Future studies can focus on the sensory properties of injera fortified with Bambara groundnut, as it plays a vital role in the acceptance of this reformulated product and studies that focus on the use of natural preservatives to extend the shelf life of baked products like injera and investigate feasible and measurable ways of extending the shelf life of injera without altering the taste nor the cost of production. Whole genome sequencing of the identified isolates can also be used to further characterise the functionality, fermentation pathways and biochemical routes during the fermentation of tef injera and injera fortified with Bambara groundnut flour. This knowledge will allow for a full exploitation of their fermentative capabilities, facilitating, at the same time the genetic manipulation of these LAB isolates. Through collaborative efforts from research institute, government officials as well as community leaders, the availability of affordable starter cultures for injera can be achieved.

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APPENDIX 1: TEF INJERA AND INJERA FORTIFIED WITH BAMBARA GROUNDNUT FLOUR (CHAPTER 4)

9% T

12% T

TIC-LA

9% TB-LA

12% TB-LAB

Tef-injera and injera fortified with Bambara groundnut flour: 9% TB; Tef injera fortified with 9% Bambara flour, 12% TB; Tef-injera fortified with 12 % Bambara flour ,TIC-LAB; Tef injera with LAB cultures, 9% TB-LAB; Tef injera fortified with 9% Bambara flour with LAB cultures, 12% TB-LAB; Tef injera fortified with 12% Bambara flour with LAB culture.

APPENDIX 2: SEQUENCE DATABASE FOR LAB AND YEAST ISOLATES

ISOLATE NO	DESCRIPTION	QUERY LENGTH	NCBI ACCESSION	REFERENCE	SIMILARITY TO GENBANK (%)
BR2	Enterobacter sp.	1407	KT957310	http://www.ncbi.nlm.nih.gov/nucleotide/94	91%
BR4	Klebsiella variicola	1400	HQ259961	http://www.ncbi.nlm.nih.gov/nucleotide/32	97%
BR10	Enterobacter cloacae	1050	CP021851	https://www.ncbi.nlm.nih.gov/nucleo	100%
BT7	Enterobacter cloacae	1050	CP021851	https://www.ncbi.nlm.nih.gov/nucleo	100
BT8	Weissella confusa	1461	GU369772	https://www.ncbi.nlm.nih.gov/nucleo	96%
BX7	Weissella confusa	1486	EU807756	https://www.ncbi.nlm.nih.gov/nucleo	97.00%
BX8	Pediococcus pentosaceus	1240	MH899319	https://www.ncbi.nlm.nih.gov/nucleo	98%
BY1	Weissella confusa	1481	KT260543	https://www.ncbi.nlm.nih.gov/nucleo	98%
BY10	Pediococcus pentosaceus	1618	MG850845	https://www.ncbi.nlm.nih.gov/nucleotide/M	100%
TF3	Lactococcus lactis	906	MK333795	https://www.ncbi.nlm.nih.gov/nucleo	99%
TF6	Weissella confusa	1509	MK680136	https://www.ncbi.nlm.nih.gov/nucleo	100%
TF8	Weissella confusa	1486	EU807756	https://www.ncbi.nlm.nih.gov/nucleo	96%
TF9	Weissella confusa	1481	KT260543	https://www.ncbi.nlm.nih.gov/nucleo	97%
TL3	Lactobacillus curvatus	1450	MH704138	https://www.ncbi.nlm.nih.gov/nucleo	96%
TL8	Weissella confusa	1499	MH819614	https://www.ncbi.nlm.nih.gov/nucleo	98%
TL9	Lactobacillus curvatus	1454	LC130555	https://www.ncbi.nlm.nih.gov/nucleo	97%
TP3	Lactococcus lactis	906	MK333795	https://www.ncbi.nlm.nih.gov/nucleo	99%
TP8	Weissella confusa	1509	MK680136	https://www.ncbi.nlm.nih.gov/nucleo	100%
BX10	Weissella confusa	1509	MK680136	https://www.ncbi.nlm.nih.gov/nucleo	99%
TF1	Lactobacillus curvatus	1454	LC130555	https://www.ncbi.nlm.nih.gov/nucleo	98%
TP10	Lactococcus lactis	1279	KX881782	https://www.ncbi.nlm.nih.gov/nucleo	97%
BT6	Lactococcus lactis	873	AB775185	https://www.ncbi.nlm.nih.gov/nucleo	100%
TP6	Weissella confusa	1509	MK680136	https://www.ncbi.nlm.nih.gov/nucleo	99%
TP2	Weissella confusa	1509	MK680136	https://www.ncbi.nlm.nih.gov/nucleo	100%
TL10	Weissella confusa	1509	MK680136	https://www.ncbi.nlm.nih.gov/nucleotide/M	99%

ISOLATE NO	DESCRIPTION	QUERY LENGTH	NCBI ACCESSIC	REFERENCE	SIMILARITY TO GENBANK (%)
BTY4	Cryptococcus albidus	893	KY106954	https://www.ncbi.nlm.nih.gov/nucleotide/KY106954.1?re	98%
BXY3	Cryptococcus albidus	893	KY106954	https://www.ncbi.nlm.nih.gov/nucleotide/KY106954.1?re	97%
TPY4	Naganishia albidosimilis	912	KY108603	https://www.ncbi.nlm.nih.gov/nucleotide/KY108603.1?re	96%
BTY1	Cryptococcus albidus	893	KY106954	https://www.ncbi.nlm.nih.gov/nucleotide/KY106954.1?re	96%
TPY1	Cryptococcus albidus	893	KY106954	https://www.ncbi.nlm.nih.gov/nucleotide/KY106954.1?re	98%
TLY3	Cryptococcus chernovii	630	AF181530	https://www.ncbi.nlm.nih.gov/nucleotide/AF181530.1?re	96%
TFY3	Cryptococcus albidus	893	KY106954	https://www.ncbi.nlm.nih.gov/nucleotide/KY106954.1?re	98%
TFY5	Naganishia albidosimilis	613	JQ916060	https://www.ncbi.nlm.nih.gov/nucleotide/JQ916060.1?re	95%
TLY1	Cryptococcus albidus	893	KY106954	https://www.ncbi.nlm.nih.gov/nucleotide/KY106954.1?re	98%
TPY1	Cryptococcus saitoi	631	EF595767	https://www.ncbi.nlm.nih.gov/nucleotide/EF595767.1?re	91%
BXY2	Cryptococcus albidus	893	KY106954	https://www.ncbi.nlm.nih.gov/nucleotide/KY106954.1?re	95%
BRY5	Naganishia friedmannii	1206	LC203718	https://www.ncbi.nlm.nih.gov/nucleotide/LC203718.1?re	97%
TLY4	Cryptococcus albidosimilis	817	JX188119	https://www.ncbi.nlm.nih.gov/nucleotide/JX188119.1?re	97%
BTY5	Cryptococcus albidosimilis	618	JQ916060	https://www.ncbi.nlm.nih.gov/nucleotide/JQ916060.1?re	95%