Synergetic Effects of *Mandillo* [*Crassocephalum macropappum* (Sch. Bip. ex. A. Rich.) S. Moore) during *Enset* (*Ensete ventricosum* (Welw.) Cheesman] Fermentation: Chemical Nature and Efficiency

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CENTER FOR FOOD SCIENCE AND NUTRITION

The undersigned have read and approved the dissertation prepared by Mr. Alemu Gonfa Robi for the fulfillment of the requirement for the degree of **Doctor of philosophy** entitled **"Synergetic effects of *Mandillo (Crassocephalum macropappum* (Sch. Bip. ex A. Rich) S. Moore) during *Enset* fermentation: chemical nature and efficiency".** Therefore, the dissertation is recommended for acceptance to the office of Graduate Studies.

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Abstract

Synergetic effects of *Mandillo* [*Crassocephalum macropappum* (Sch. Bip. ex. A. Rich.) S. Moore] during *Enset* fermentation: chemical nature and efficiency

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Enset (*Ensete ventricosum* (Welw.) Cheesman) is an ornamental plant in Asia, widely distributed in Africa and cultivated only in some regions of Ethiopia. *Enset* plant withstands prolonged period of drought and known for its high yielding potential. Fermentation of *Enset* produces a starchy food named *Kocho*, however, it is not practiced extensively. The principal reasons for low popularity of *Enset* are its uncommon sensory attributes among non-*Enset* consuming people, short shelf-life, nutrient losses, long fermentation period and lack of awareness. In Ethiopia, the Shekacho society adds *Mandillo* (*Crassocephalum macropappum* (Sch. Bip. ex. A. Rich.) S. Moore) stem during traditional *Enset* fermentation without knowing its scientific significance to the fermentation processes. Thus, the present study was initiated with the main purposes of investigating the indigenous knowledge of Shekacho society in *Enset* cultivation, *Kocho* consumptions, bioactive components of *Mandillo* parts and to determine its effects in *Enset* fermentation processes. The study was conducted using standard methods. To evaluate the indigenous knowledge of Shekacho society, analytical data were collected using semi-closed questionnaire, field observations, interviews and discussion with key informants with local annotations review. *Enset* plant physical structures were assessed. Phytochemical composition of *Mandillo* was evaluated. Its DNA damage protective and antioxidant activities were analyzed. Antimicrobial activity of *Mandillo* was tested against standard bacterial and yeast strains. Cytototoxicity of *Mandillo* was also evaluated. Fermentation process was carried out from 0-90 days. The pH value, titratable acidity, lactic acid and acetic acid contents were measured. Microbial counts were made on their respective agar media. Lactic acid bacteria (LAB) and yeast isolates were identified using morphological, biochemical and molecular techniques. Proximate composition, mineral contents, phytic acid, tannin, water holding capacity, and sensory attributes

of *Kocho* were evaluated and the main findings are described as follows. The descriptive analysis of the data and the corresponding results indicated the existence of a high degree of consistency among the respondents with regard to *Enset* histories, fermentation processes, productivity, shelf-life of *Kocho* and food preparation. About 115 *Enset* cultivars were identified. Phytochemical analysis of *Mandillo* showed the presence of saponins, tannins, anthraquinones, steroids, terpenoids and flavonoids. Total phenolic and flavonoid contents of the aerial part calculated as 101.48 mg GAE (Gallic acid equivalent)/g and 293.25 mg QE (Quercetin equivalent)/g, respectively. *Mandillo* showed strong 2, 2-diphenyl-1-picryl hydrazyl (DPPH) scavenging potential with $IC_{50} \leq 50$ µg/mL. The stem of *Mandillo* showed promising protective activity against oxidative DNA damage. The minimum inhibitory concentration of stem against *E. coli* KCTC 1682 and *Candida utilizes* KCCM 11355 was ≥ 15.62 µg/mL. The final pH of *Kocho* fermented with starter culture but no *Mandillo* (Koki) and with *Mandillo* (Kom) decreased to 4.49 and 4.10; respectively, which are significantly different and didn't allow the growth of spoilers in Kom. Titratable acidity, lactic acid and acetic acid contents of Kom increased by 64.34%, 103.90% and 40.04%, respectively when compared to Koki. Koki and Kom samples had LAB counts of 1.16×10^7 and 2.83×10^8 CFU/g, respectively, on the day 37. Treatments with *Mandillo* stem significantly ($p < 0.05$) lowered counts of aerobic mesophilic bacteria, aerobic spore formers and coliforms. Staphylococci were below detectable level. The 16S rRNA gene sequences analysis and phylogenetic tree reconstruction identified LAB species as *Lactobacillus plantarum* (69.37%), *Leuconostoc mesenteroides* (9.91%), *Lac. paracasei* (9.01%), *Lac. brevis* (3.60%) and other rare species. *Lac. paracasei* and *Lac. brevis* were dominantly found in Kom during the final days of fermentation. An 18S rRNA gene sequences evaluation identified yeast isolates as *Candida boidinii* (30%), *Wickerhamomyces* sp. ES22S03 (16%), *Pichia fermentans* (16%), *Williopsis* sp. (10%) as dominate species and others minor strains. *Kocho* composition analysis showed significant ($p < 0.05$) differences between Koki and Kom in moisture contents (8.34 to 8.80%) and (9.14 to 7.95%), fiber (7.92 to 5.56%) and (6.25 to 3.96%), protein (3.24 to 3.68%) and (3.59 to 3.95), phytic acid (5.83 to 4.11 mg/g) and (4.95 to 4.33 mg/g), water holding capacity (8.55% and 6.37%); respectively. Mineral contents of Koki and Kom also calculated as Ca (9.84 mg/100 g and 10.08 mg/100 g), Fe (2.12 mg/100 g and 2.08 mg/100 g), Zn (1.99 mg/100 g and 1.77 mg/100 g) and Cu (0.12 mg/100 g and 0.16 mg/100 g); respectively. Sensory evaluation revealed overall acceptability values of 73.27% (Koki) and

97.20% (Kom). Cytotoxicity evaluation of *Mandillo* stem against Raw Cells 264.7 showed no toxicity at ≤ 4 µg/mL. From the results of the descriptive analysis of the data and the corresponding results suggested by the respondents, it is understood that *Enset* is very important for food security and environmental protection. Phytochemical contents and activities of *Mandillo* revealed consistency with the starter culture role played by the addition of the stem part of the plant. The effect of adding *Mandillo* during *Enset* fermentation showed significant differences in all of the measured *Kocho* quality parameters, food composition, microbial dynamics and diversities and sensory attributes of experimental *Kocho* product compared to the control sample. In general, improvements of the basic components of *Kocho* dough are responsible for the enhancement of its shelf-life, safety and sensory attributes of the food as a result of good fermentation processes. In the light of the current finding, extensive investigation are required to qualify *Mandillo* as food preservative and shelf-life enhancer for its application in food sectors.

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Dedication

This research work is dedicated to my beloved wife, Kelemework Dessalegn who has fully shouldered the burden of looking after our children and me in this long journey of my study period.

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Acronyms and Abbreviations

AA	Acetic acid
AOAC	Association of Official Analytical Chemists
ATCC	American Type Culture Collection
CFU	Colony forming unit
DAD	Diode array detector
DCM	Dichloromethane
DMEM	Dulbecco's modified Eagle medium for growth of Raw Cells 264.7
DPPH	2, 2-diphenyl-1-picryl hydrazyl
DW	Distilled water
FBS	Fetal bovine serum
GAE	Gallic acid equivalent
HPLC	High performance liquid chromatography
IC50	Inhibitory concentration
ITS1	Internal transcribed spacer (forward primer) for yeast
ITS4	Internal transcribed spacer (reverse primer) for yeast
KCCM	Korea Culture Center of Microorganisms
KCTC	Korean Collection for Type Culture centers
LA	Lactic acid
LAB	Lactic acid bacteria
MB/FC	Minimum Bactericidal/Fungicidal Concentration
MEGA6	Molecular Evolutionary Genetics Analysis version 6.0
MHB	Muller Hinton Broth

MIC	Minimum Inhibitory Concentration
MRS agar	de Man, Rogosa, and Sharpe agar,
MSA	Mannitol salt agar
MTT reagent	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent
NJ	Neighbor-Joining
OD	Optical Density
PCA	Plate count agar
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
QE	Quercetin equivalent
SD	Standard Deviation
TA	Titratable acidity
VRBA	Violet red bile agar

Meanings of special terms and words

1492R	Reverse primer
27F	Forward primer
Aaco	Seedlings of *Enset* plant
Amicho	Potato like food obtained from underground corm of *Enset*
Atinaa'o	Male types *Enset* plant
Bacco	*A* mixture of *Bulla and Kocho*
Baqqo	Division of *Enset* corm into parts
Baqqoo	Inner soft part of pseudostem
BOH	Butanol
Buddeno	Ethiopian *Injera*
Bulla	Starchy foods obtained as by product of *Enset* fermentation
Buuxo	Porridge prepared from *Kocho*
Dooloo	Bucket like material made from bamboo
Dubbo	Traditional dress for woman, made up of *Enset* fiber
Dubo	Interlocking leaf sheath of *Enset*/pseudostem
Duqquushe-waamo	Leaf of *Allium sativum*
Enset	*O*rder *Scitamineae*, a family of *Musaceae*, genus *Ensete*, known as false banana
Fussoo	Traditional dress for woman, made up of *Enset* fiber
Gaamo	Suckers of *Enset* plant
Gamoo	*Enset* fermentation pits
Gichoo	Ornamental made from *Enset* fiber put on the shoulder by male

Ittino	*Bulla*
Kaakiro	Dried leaf sheath of *Enset*
Kefo	*Sacred basil (Yehabesha)*
Kiino	Microorganism that causes wilting of *Enset* plant
Kiisho	Starter culture for *Enset* fermentation
Kiisho-goono	Bowel shaped structures made from underground corm for starter culture preparation
Ko	Initiation period *Kocho* sample
Kocho	A woman dress made from *Enset* fiber
Kocho/Qoocoo	Starchy foods obtained through *Enset* fermentation processes
Koki	Control group *Kocho* samples
Kom	Experimental group *Kocho* samples
Komixoo	Wooden grater for underground corm pulverization
Kujjo	Soup prepared from *Bulla*
Lac.	*Lactobacillus*
Leu.	*Leuconostoc*
Maache	Female type *Enset* plants
Maaroo	A scraper made from bamboo, rib bone and wood
Maato	*Enset* leaf
Mandillo	A family of *Asteraceae*, genus, *Crassocephalum*, specific epithet, *macropappum*
Matoo	Wooden board uses for *Enset* scraping
Maxino	Pancake prepared from *Kocho*

Mundriiqqo	*S*hoot part of *Enset*
Ooqqo	*Enset* leaf *m*idrib
Quuro	*S*queezed liquid of *Kocho*
Raw Cells 264.7	The cell used for cytotoxicity test
Shikkoo	Knife
Shitoo	Pre-fermented lower part of *Enset* leaf midrib
Shoobbo	*Lippia adoensis*
Tokko	Final stage of *Enset* seedlings transplantation
Toocho	*Cymbopogon citratus*
Uukko	Further propagation of *Enset* seedlings
Uuxo	*Amicho* or underground corm.
Yi’o	Fiber
Yo’o	Leaf sheath

Chapter 1

1. Introduction

1.1. Background

Food is a source of nutrient, energy, satisfaction and wholeness (Jennifer and Harrell, 2015; Onwordi *et al*., 2009), obtained from plant or animal sources. Food may be maintained through food security such as availability, accessibility, safety and utilization of the nutrients (Fila *et al*., 2013). Food has behavioral, cultural, religious, social, psychological, environmental, moral, medicinal, nutritional, taste preferences, habitual and gender related backgrounds (Olsen, 2007). For instance, the most prestige food in one culture is immoral to be consumed in another culture or it may not be ritual in some other religions (Crapo, 2001). Food taboo and lack of information have their own contributions to the food insecurity (Nakashima, 2000). High population density and land mismanagement are also the most perilous aspects of food deficiency (FAO, 2009). As the world's population have been alarmingly increasing (Lamichhane, 2014; Cohen, 2006), food security, land management and environmental protection are becoming serious agendas of the century (Pender and Gebremedhin, 2007; Acquay, 2003; Pretty *et al*., 2003).

To accommodate such huge number of population in resource limited and dynamic world (current total population 7,238,184,000, birth rate 143,341,00/year and death rate 56,759,000/year) (PRB, 2014), scientists have been looking for different means of survival, like family planning, increase in production and productivity (Babu, 2000). Some of the means for product maximization include application of modern technologies and research outputs (Acquay, 2003). On the other hand, the society has its own social, psychological, religious and moral backgrounds and the right to maintain their security, adjustment with the new technologies and

findings. For instance, it is instructional and lawful for religious people to consume food from animal source selectively. It is also equally valued to increase in number, keep the earth developed, maintained and protected (Genesis, 1:28). As it is written in the book of Genesis (1:28), God blessed the human beings and said to them, *"Be fruitful and multiply and fill the earth and subdue it and have dominion over the fish of the sea and over the birds of the heavens and over every living thing that moves on the earth.* In Genesis (1:29), God also said, *"Behold, I have given you every plant yielding seed that is on the face of all the earth, and every tree with seed in its fruit, you shall have them for food."*

Even though, plants convert chemical compounds to carbohydrate by the use of solar energy, yet it is not economical to produce carbohydrate from the constituents in industries by applying different source of energy (Varghese *et al*., 2009). Therefore, to accommodate the increasing number of the world's population, within the available areas of the land, among other alternatives, production of high yielding, drought tolerant and disease resistant plant species is a wise choice (Kennedy and Burlingame, 2003; Pretty *et al*., 2003; McNeely, 1994).

Accordingly, to realize food security, there are ample opportunities around the world, as the world is full of diversified high yielding potential plants (Acquay, 2003). One of the high yielding potential plants in Ethiopia is *Enset* (*Ensete ventricosum* (Welw.) Cheesman) and Cassava that yields more than 300 and 250 quintal/hectare/year, respectively (Onyenwoke and Simonyan, 2014; Tsegaye and Struik, 2001), however, the shelf-life of *Kocho* is by far better than Cassava if handled properly. Due to its drought tolerance and high productivity, *Enset* is regarded as a priority food security crop in Ethiopia (Habte, 2013; Tadele, 2009), where it makes a major contribution to food security of the country. *Enset* is also known by the name of “Tree against hunger” (Stone *et al*., 2011). For instance, regions where *Enset* is used as staple food are

usually less affected by the recurrent drought periods that occur in other parts of Ethiopia (Mohammed, 2013; Birmeta *et al*., 2004). This fact may be justified using comparative explanation to the productivity of common crops and cereals in the country. By the use of modern agricultural packages, the productivity of cereals and corns are not more than 80-100 quintal per hectare per year (Rockstrom *et al*., 2009), while the productivity of *Enset* was found to be about three times higher than the yield of the aforementioned crops and cereals (Tsegaye and Struik, 2001). This implies that with the existing life status and technology, specifically Ethiopia and the world in general may accommodate more than threefold of the existing number of their populations.

The main food items obtained from *Enset* are *Kocho, Bulla* and *Amicho. Kocho* is more fibrous, major product of *Enset* fermentation and a starchy mineral rich food. *Bulla* is less fibrous, unfermented, partially fermented or sometimes fermented byproduct during *Enset* fermentation (Ashenafi, 2006), where as *Amicho is* unfermented and underground part of *Enset* (the corm) (Karssa *et al*., 2013; Hunduma and Ashenafi, 2011; Ashenafi, 2006; Gashe, 1978a). All the three parts of the food items are rich in starch and mineral contents (Tadele, 2009; Ashenafi, 2006), even though less in protein, fat and vitamins (Hunduma and Ashenafi, 20011; Gashe, 1987a).

In spite of the fact that *Kocho* is very important source of food, energy and industrial raw material (Olango, 2009***)***, in Ethiopia, its usage is known by insignificant regions of the country as only 67,000 sq. km of the total land (about 1.2 millions of sq. km) was cultivated by *Enset* plant (Ashenafi, 2006). The main reasons to limited cultivation and consumption of *Kocho* could be related to uncommon inherent sensory attributes for non-consumers, nutrition loss, long fermentation period, lack of awareness and its short shelf-life. Sensory attributes of *Kocho* are not acceptable by most of none *Enset* producing areas since they are not familiar to the odor,

taste and flavor of the food. Most of the uncommon sensory attributes are the results from microbial spoilage due to high moisture content of *Kocho*. High moisture content supports the growth of spoilage microorganisms which in turn produce unpleasant organic compounds. The nutritional and organoleptic qualities of *Kocho* could therefore, be process related. Nutrient loss and time taking fermentation processes are common and vary from place to place (Ashenafi, 2006; Tsegaye and Struik, 2001). Food taboo, lack of knowledge, experience, skills and technology in *Enset* cultivation, fermentation and consumption are the other drawbacks (Stone *et al*. 2011).

To improve *Kocho* related problems and increase its productivity, shelf-life, food safety and quality of the products, among numerous techniques, fermentation methods (Marshall and Mejia, 2012) and addition of traditional preservatives of plant sources (Sahlin, 1999; Stuart, 1986***)*** are very important activities. Such traditional preservatives contain chemical constitutes with characteristic of flavors, antioxidant as well as antimicrobial activities (Smid *et al*., 1999). In Ethiopia different societies were exercising the use of a number of diversified plant spices into food products to increase shelf-life, safety and improve organoleptic qualities (Nakashima, 2000).

When practices of *Enset* fermentation and usage of *Mandillo* (family of *Asteraceae*, genus: *Crassocephalum*, specific epithet: *macropappum* (Sch. Bip. ex. A. Rich.) S. Moore) as a traditional fermentation enhancer came to fore front, the indigenous knowledge of Shekacho society that attracted the attention of the investigators. For instance, according to the indigenous knowledge of Shekacho society (‘traditional wisdom’), the application of *Mandillo* as ingredient of starter culture in *Enset* fermentation processes to enhance fermentation rate, improve qualities of sensory attributes and increase shelf-life is a very important research area.

Various research findings were reported by different investigators on *Enset* with the major concerns such as *Enset* cultivation and productivity (Tsegaye and Struik, 2001), food safety and security issues (Kanshei, 2002). Moreover, Microbial dynamics of *Enset* fermentation (Gashe, 1987a), microbial spoilage and accompanying changes (Gashe, 1987b) and biochemical changes during fermentation and the effect of altitude on microbial successions (Hunduma and Ashenafi, 2011) were evaluated. Chemical composition and degradability in different morphological fractions (Nurfeta *et al.,* 2008), mineral content (Atilabachew and Chandravanshi, 2008) and mineral absorption inhibitory factors (Umeta *et al.,* 2005) were assessed. Improving the indigenous processing of *Kocho* using different cultivars of *Enset* (Yirmaga, 2013), differences between the pits and jars fermentation of *Kocho* (Karssa *et al.,* 2013) were also investigated. However, studies on the traditional *Enset* fermentation using *Mandillo* stem to improve the uncommon *Kocho* quality parameters that enhance the fermentation rate and improve nutritional profile in general, have not been conducted. There were also scarcity of literature on phytochemical profile, antioxidant capacity, antimicrobial activities and cytotoxicity of *Mandillo*. Furthermore, there were no data on proximate compositions, mineral contents, water holding capacity and anti-notional factors of *Kocho* from the research area. The current study was, therefore, initiated to bridge the aforementioned knowledge gaps.

1.2. Objectives

1.2.1. General objectives

The general objectives of this study were to investigate the indigenous knowledge of Shekacho society and the synergetic effects of *Mandillo* during *Enset* fermentation

1.2.2. Specific objectives

The specific objectives of the present study were to:

- investigate the indigenous knowledge Shekacho society
- sort out the existing *Enset* vernaculars
- evaluate the chemical nature of *Mandillo*
- analyze the activities of *Mandillo*
- evaluate cytotoxicity level of *Mandillo*
- evaluate the effects of *Mandillo* during *Enset* fermentation
- analyze *Kocho* composition
- evaluate sensory quality attributes
- create awareness on the major advantages of *Enset* cultivation and *Kocho* consumption

1.3. Significances of the study

Despite the fact that *Enset* is a very important source of food, industrial raw materials and multipurpose plant, production and consumption of *Kocho* are limited to insignificant areas of Ethiopia. Therefore, the present study is irreplaceably significant to address the issues of

drought tolerance and high productivity of *Enset* as significant for environmental protection. It serves for food security and utilitarian to nourish the society with densely populated areas of the country.

The outcomes of the research carried out would have great significances for awareness creation to the non-*Enset* cultivating area on the multidimensional uses of *Enset*; for instance, the nutritional contents of *Kocho*, including the high carbohydrate, mineral and fiber contents and

diversified beneficial microorganisms having numerous health benefits and contributions to food security. Fermentative LAB could be beneficial source of probiotics on *Kocho* traditional recipes, in presence of *Lac. plantarum.*

It is also significant to introduce the indigenous knowledge of the Shekacho society to the world in *Enset* fermentation and the use of *Mandillo* to enhance the fermentation speed and product (*Kocho*) qualities which has to be adopted throughout the country in particular and possibly the world in general. Such synergetic effects would make new grounds for further research in antioxidants and phytochemical of *Mandillo,* that is otherwise neglected vastly unexpected.

Chapter 2

2. Literature review

2.1. Background history of *Enset*

Enset (*Ensete ventricosum* (Welw.) Cheesman) belongs to the order *Scitamineae*, the family *Musaceae*, and the genus *Ensete*, known as false banana, which is widely distributed in Eastern and Southern Africa (Bender, 2006). It is cultivated in southern, southwestern and some parts of Oromia National Regional State in Ethiopia (Olango *et al*., 2014; Hunduma and Ashenafi, 2011; Kanshie, 2002; Brandt *et al*., 1998). The plant grows fairly well between 1100-3000 m above sea level (Brandt *et al*., 1998). A vigorously growing healthy plant can withstand prolonged periods of drought (Gashe, 1987a). *Enset* is a perennial monocarpic plant with leaves, pseudostem and a large underground corm (Kanshie, 2002). As it was stated by Nurfeta *et al*. (2007) and Karin *et al.* (2005), the criteria for differentiation of the phenotype are the size, color and shape of pseudostem and leaves, and color of leaf sheaths, midribs, and lamina.

2.2. Uses of *Enset*

Enset is the main source of food in the densely populated areas of the central and southwestern parts of Ethiopia (Ashenafi, 2006; Tsegaye and Struik, 2001). Out of more than 90 million, *Enset* is a staple food for about 15 to 20 million people of Ethiopians in mixed subsistence farming systems (Olango *et al*., 2014; Kanshie, 2002; Zippel and Karin, 2002). All parts of *Enset* plant are important to provide useful products for household, agriculture, food, construction, animal feed and traditional medicinal treatments (Nurfeta *et al*., 2007; Karin *et al*., 2005; Kanshei, 2002). *Kocho* and *Bulla* are made to fermented bread or porridge like foods that consumed locally as well as exported to urban markets, while *Amicho* is eaten boiled like potato (Brandt *et*

al., 1998). Concerning the health benefit, because of the existence of *Lactobacillus plantarum* and other microbial populations in fermented *Kocho, it* can even have beneficial health effects, when these fermenting microorganisms possess probiotic activity (Molin, 2001; Gashe, 1987a).

2.3. *Enset* productivity

Leaf sheaths and the corm of *Enset* provide starchy foods namely, *Kocho*, *Bulla* and *Amicho* (Gashe, 1987a).

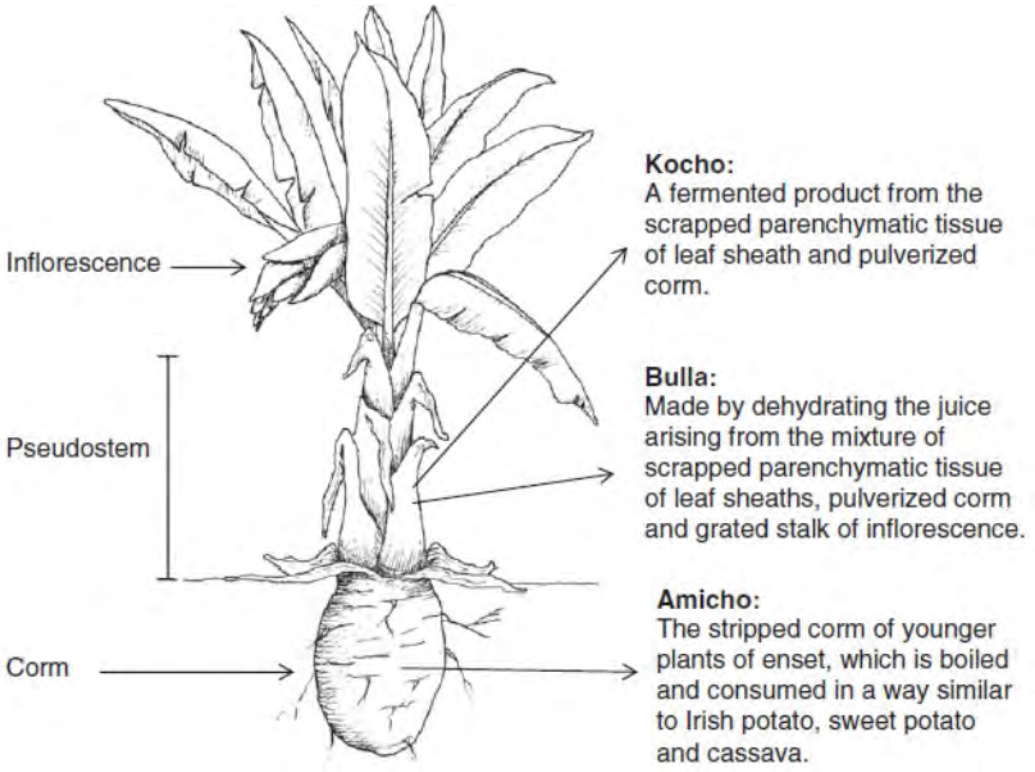


Figure 2.1: Morphology of the *Enset* plant and its major food products (Forsido *et al*., 2013).

Kocho and *Bulla* can be stored for long periods of time after fermentation (Ashenafi, 2006; Gashe, 1987b). According to Tsegaye and co-workers (2001)**,** *Kocho* yield of *Enset* per unit space and time, in terms of edible dry weight and energy, was much higher than the yields of any

other starch rich crop cultivated in Ethiopia and estimated to range from 17.1–33.9 ton ha^{-1} $year^{-1}$. *Enset* provides a long term sustainable food supply with minimal inputs (Lost Crops of Africa, 2006). As a result of such properties, *Enset* has been regarded as a priority food security crop in Ethiopia (Karssa *et al*., 2013; Tadele, 2009). For instance, regions where *Enset* is used as staple food are usually less affected by the recurrent drought (Mohammed, 2013; Birmeta *et al*., 2004). *Enset* is also known by the name of "tree against hunger" (Stone *et al*., 2011).

2.4. Fermentation processes of *Enset*

Food fermentation is one of the oldest known uses of the traditional biotechnologies (Ikpeme-Emmanuel, 2011; Campbell-Platt, 1994), that food and beverages are defined as products obtained through desirable biochemical changes caused by the action of microorganisms or enzymes (Ashenafi, 2006). All over the world, the foods and beverages continue to constitute an important part of human diet and are estimated to provide some 20–40% of our food supply worldwide (Lei, 2004; Campbell-Platt, 1994). Particularly in developing countries, where refrigeration is not always an option, the fermentation process is widely used and of crucial importance to prolong the shelf-life of foods in addition to improving the nutritional value and reducing the risk for foodborne illnessies.

Traditional fermentation of foods is also important to preserve nutrients, provide diversified flavors, aromas and acceptable texture, which enrich the human diet (Karovicova and Kohajdova, 2003). Fermented foods attain improved taste, nutritional value and digestibility (Adesulu and Awojobi, 2014; Oyewole and Isah, 2012; Rhee *et al*., 2011). The other benefits of locally fermented foods are enhancement of organoleptic properties, provision of nutritional quality, detoxification, and production of antibiotics which have large contribution for food

security (Uddin *et al.*, 2014). Fermenting microorganisms can synthesize certain amino acids and improve protein quality and availability of B group vitamins. Fermentation also results in reduction in ant-nutrients like physic acid and tannin contents, which may increase the amount of soluble iron, zinc and calcium in several folds (Blandino *et al*., 2003 and Henry, 2001).

Fermented foods have many other health benefits; for instance, the antimicrobial property of lactic acid bacteria in fermented herbs increases by about 80%–170% compared to the crude herbal extract (Vaishnavi, 2010***).*** The author further noted that herbals have selective inhibition potential and can selectively suppress common human pathogens like *Escherichia coli, Staphylococcus aureus, Salmonella typhi, Psuedomonas aeruginosa*, *Bacillus cereus*, *Bacillus subtilus, Klebsiella* species and many others.

Microorganisms in different fermented foods and areas may be different. Accordingly, yeast strains used for leavening are different in different situations depending on physical and chemical environment. Some of the known yeast strains, *Saccharomyces cerevisiae*, *Kazanchastaniaexigua*, *Candida milleri* and *Candida humilis* are amongst the common one (Zhou, 2012).

In South Korea and Philippine, *Sikhae* and *Burong*, respectively made by mixing salted fish and cereals of which fermentation is initiated by *Leu. mesenteroides* (Rhee *et al*., 2011). LAB strains in Korean Kimchi, *Leu. mesenteroides, Lac. plantarum* and *Lac. kimchii* were found to be dominant microorganisms (Wadamori, 2014; Yoon, 2000). In all of these cases, pathogenic bacteria and spoiler species may be reduced or inhibited by bioactive products and reduction of pH during the fermentation course (Onda, 2003).

The diversified beneficial microorganisms have synergetic effects in inhibition of foodborne pathogenic and spoiler microorganisms. It is also equally important for the health benefits of human being by producing numerous substrates with medicinal and nutritional values which make fermented food wholesome. When food safety is also a major concern, antimicrobial substances as bacteriocins produced by some of LAB can also play a great role.

2.5. Microbial activities in *Enset* fermentation processes

According to Gashe (1987a), on the day zero, *Kocho* has high moisture content, low titrateble acidity, near neutral pH and high soluble reducing sugar concentration when compared to the final fermentation days of *Kocho*. During the initial period, *Kocho* contained a diverse group of microorganisms such as aerobic and anaerobic spore formers, Gram negative bacteria including members belonging to the *Enterobacteriaceae*, lactic acid bacteria and yeasts. In indigenous fermented foods, the microorganisms responsible for the fermentation are usually the microbial flora naturally present on the raw substrate (Ashenafi, 2006; Gashe, 1987a).

It has also been indicated that *Leuconostoc mesenteroides* is responsible for initiating the fermentation of *Enset* during initiation period. As it was described in the previous study, because of the activities of this species and to some extent, of *Streptococcus faecalis*, the pH of the fermenting *Kocho* was reduced from 6.5 to 5.6 (Gashe, 1987a). These organisms may be then succeeded by some of the homofermentative *Lactobacillus* species (Gashe, 1987a). Through the activities of the *Lactobacillus* species, the pH can be further reduced to 4.2 (Gashe, 1987a). The microorganisms are also temperature dependent. For instance, if *Pediococcus cerevisiae* present in *Kocho*, it can't achieve prominence in relatively low fermentation temperature between 14°-18°C (Gashe, 1987a). Spore-formers may be present in fairly high numbers during the first 15

days of fermentation (Gashe, 1987a). The butyrous odor usually detected during the first two weeks in fermenting *Kocho* is due to the activities of certain clostridial species, and yeasts can be also present in fairly high numbers (Gashe, 1987a).

2.6. Shelf-life of *Kocho*

When microorganisms utilize food nutrients for growth and produce some end products or changes that are undesirable for humans will take place. These changes are said to spoil the products and that might be the end of the shelf-life. Spoilage may be result in changes of flavors, odors, colors or textures of the food (FAO, 1998). Spoilage is not necessarily hazardous to humans but does cause food loss and is an economic burden (Abbas, 2009; Doyle, 2007). Substantial yield losses do occur due to fungi, bacteria and viral contamination and are of increasing worth as to food production needed for the vast expansion of world population. The activity of the microbes can cause undesirable effects in grains including discoloration, contribute to heating and losses in dry matter through the utilization of carbohydrates as energy sources, degrade lipids and proteins or alter their digestibility. These in turn produce volatile metabolites giving off-odors, cause loss of germination, baking and malting quality and affect use as animal feed and food or as seeds.

Likewise, in *Enset* fermentation processes, the loss of nutrient value of *Kocho* due to spoilage microorganisms is occasionally high in pits, where there are air pockets or where anaerobic conditions have not been maintained (Gashe, 1987b). The spoilage organisms associated with *Kocho* originate from the action of bacteria, yeasts and molds on foods and from chemical changes within the food and bring about softness, sliminess and discoloration (Gashe, 1987a). To increase the shelf-life, *Kocho* could be left in a storage pit for 15 to 30 days during fermentation,

and it can also be stored for many months (3-6) and even for several years under anaerobic condition (Gashe, 1987a).

2.7. Food preservatives

Food preservation usually involves preventing the growth of bacteria, fungi/yeasts, or other microorganisms as well as retarding the oxidation of fats that cause rancidity. It may also include processes that inhibit visual deterioration, such as the enzymatic browning reaction like in apples after they are cut during food preparation. Thus, at present, in the time of agro industrialization, many interventions must be used to prevent undesirable changes in food products, such as creation of closed environment, killing or inhibition of the growth of foodborne pathogens and spoilage microorganisms by the use of different preservatives in food industries and at household level (Sagdic and Ozcan, 2003).

Many food preservation processes are followed via different designed, large number of methods which may be classified as either physical means, use of synthetic chemicals, biological methods or application of natural products (Pace *et al*., 1989). For example, preserving different food spoilage by physical means includes turning it into jam, involves boiling to reduce the moisture content, deactivation of enzymatic activities, sugaring to prevent bacterial re-growth and sealing within an airtight materials to prevent recontamination, freezing, pasteurization and sterilization. Killing or inhibition of bacteriai growth by synthetic chemicals is another option of food preservation. Another methods of preservation work may take place by introducing benign bacteria or fungi to the food items. However, creating acceptable nutritional value, texture and flavor by the use of natural products is an important aspect of food preservation. Natural antimicrobials produced during fermentation are important and safe food preservatives which

include bacteriocins, organic acids, alcohols, essential oils and phenolic compounds (Rupasinghe and Yu, 2012; Ofor and Onomerhievurhoyen, 2011).

It is reported that synthetic chemicals with antioxidant and antimicrobial properties are often used as preservative in food processing and storage to inhibit food borne pathogens and spoilers to extend shelf-life. However, the safety of most of these synthetic antioxidants and antimicrobial chemicals has been doubted due to toxicity, organ damages and carcinogenicity. Consumer awareness and concerns over these potential risks of synthetic food additives to human health have renewed the interests in using biological and naturally occurring alternative preservatives (Dua *et al*., 2014).

The health status of human being partly lay in some chemical substances of plant sources that may produce a definite physiological action on the human body (Oloyede and Ogunlade, 2013; Ooi *et al*., 2012; Wu *et al*., 2009**)**. The most important of these bioactive constituents of plant sources are alkaloids, tannins, flavonoids, phenolic compounds, saponins, anthraquinones, glycosides, steroids, cardiac glycosides and many other bioactive compounds have incredible importance as drugs and food ingredients in human life (Oloyede and Ogunlade, 2013; Wu *et al*., 2009).

According to Proestos *et al*. (2013) and Suhr (2003), applying relevant plant products in food processing is recommended to minimize the undesirable effects of synthetic food preservatives in human health. Aromatic plants are well known for their antioxidant and antimicrobial properties (Charalampo *et al*., 2013; Wu *et al*., 2009; Smid *et al*., 1999) that prevent food degradation and alteration as they are rich in phenolic substances, usually referred to as polyphenols, which are ubiquitous components of plants and herbs. It was further justified by Proestos *et al*. (2013) and

Alsabri (2012) that secondary metabolites of plant materials (polyphenols) are potential antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. A strong positive correlation between antioxidant activities and contents of phenolic acids was also studied by Vladimir-Knežević *et al*. (2011) that antioxidants can delay, inhibit or prevent the oxidation of oxidizable fractions materials by scavenging free radicals. Antioxidants may also play a role in health benefits, offering an opportunity to add flavor without adding extra ingredients with health drawbacks like excess fat or salt (Wu *et al*., 2009).

2.8. Antimicrobial activities of natural products

A number of scientists have reported that antibacterial activities of extracts of different plants against various microorganisms and medicinal values of herbs have been assessed (Ahmad and Beg, 2001). Some spices were also specifically tested for antimicrobial activities (Shelef, 1983; Sagdic *et al*., 2003). Antimicrobial activities of plant parts may be evaluated by different methods like quantitatively by agar and broth dilution methods (Mohammadzadeh, 2012; Wiegand *et al*., 2008; Barry, 2001). The aim of broth dilution methods is to determine the lowest concentration of the assayed antimicrobial agent by minimal inhibitory concentration (MIC), that under defined test conditions, inhibits the visible growth of the bacterium being investigated (Wiegand *et al.*, 2008).

2.9. Natural products as food preservatives and enhancers

In Ethiopia, there are also different plants parts important as foods to be consumed directly, ingredients of food as preservatives, or as flavoring agents to enhance organoleptic properties of

food stuff or they may be used in food processing practice in traditional ways or in modern industries.

Even though, spices and herbs are essential components of most of the traditional food since ancient times (Sahlin, 1999; Stuart, 1986), there are little reports on some of the spices and herbs (Romson *et al*., 2011; Singh *et al*., 2002). According to Bekele (2007), there are large numbers of moderate to high value medicinal plants, herbs and spices existing in Ethiopian wild. Despite the existing medicinal herbs and spice, plants only small percents are traded.

Among the important food preservatives in Ethiopia, *Mandillo* stem is one of the herbs added to *Enset* fermentation processes that believed to shorten the fermentation period, improves sensory qualities attributes and increase shelf-life of *Kocho* that has been in use by Shekacho society for long period of time (Gonfa *et al*. Unpubl.)

2.10. Background history of *Mandillo*

Mandillo (Crassocephalum-macropappum (Sch. Bip. ex A. Rich.) S. Moore) is endemic plant to Ethiopia. It is common in moist places along margins of evergreen forest, dry evergreen woodland and bush land, waste land and along roadsides; 1600–3270 m. The plant may be found in Gonder, Gojam, Wello, Shewa, Wollega, Illuu-Abbaa-Booraa, Keffa, Gamo-Gofa, Sidaamaa, Bale and Harerge; not known elsewhere (Tadesse, 2004).

The structures of the plant were described as a straggling perennial herb, 0.5–2 m long. Stem is slender, striate-sulcate, scabrid-pubescent, and weak and often growing through herb or shrub clump. Leaves are simple, ovate, pale green above, grey-green beneath, 2–10 x 2–10 cm, sparsely to densely scabrid-pubescent, especially along the veins, margins closely, irregularly and often unequally serrate, base truncate or cordate, apex shortly acuminate or apiculate,

petiolate, petiole 1–7 cm long, sparsely to densely pubescent, auriculate at base. Capitula is discoid, campanulate-hemispheric, 10–12 x 6–8 mm at anthesis, solitary or a few together on long peduncles. Peduncle is sparsely to densely pubescent, bracteate near the capitula. Receptacle is flat or slightly convex, 5–8 mm wide in fruit. Involucre 8–10 x 6–8 mm at anthesis. Calyculus is linear, 18–22, ciliate, 4–5 x 0.5 nun. Phyllaries are green at anthesis, linear, glabrous, apex dark brown to blackish, 6–8 x 1 mm. Disc florets bright yellow. Corolla is 7–9 mm long, 5–10 bed. Cypselas is black, ribbed, pubescent between the ribs, 1.8–2 x 0.4–0.6 mm. Pappus white, 9–10 mm long (Tadesse, 2004). According to Biru (2003), *Mandillo* is an herbaceous plant, found in Beteyu forest $8^0$13'N and $38^0$21'E near Butagira, Ethiopia. *Mandillo* was recorded in all transects in Bonga forest ***(***Nune, 2008***).*** It is also practically observed in Sheka Zone (SNNPRS), around Gordomo and Gore, Iluu-Abbaa-Booraa Zone, Oromia National Regional State, Ethiopia, during field trips of the research work.

Figure 2.2: *Mandillo* (*Crassocephalum macropappum*); discovered by *Schimper (1840)* in Ethiopia.

Chapter 3

3. Materials Subjects and Methods

3.1. Materials

To accomplish the intended research work, the materials and instruments used include: atomic absorption spectrophotometer (AAS) Shimdzu (AA-2600, Japan); cellulose acetate membrane with a pore size of 0.45 µm (Millipore HAWP 02500); drying oven (DHG-9055A); electric grinder (FM 100 Model, China); Enzyme Linked Immunosorbent Assay (ELISA) micro plate reader (BioTake, PowerWave , Korea); gaspak jars (GasPak System, BBL); HPLC, (Agilent Technlogies 1200 series, Germany); light microscope (magnification power of up to 1000×); low speed centrifuge model 800 (China, Beijing); muffle furnace, Eurotherm (CSF 1200); orbital shaker (SSL1); rotary evaporator (RE300); SensION MM 150 pH meter (MACH Switzerland); SolGent's PCR series (Korea); spectrophotometer lambda 950 (PerkinElmer, Liantrisant, CT728YW UK CoO GB); ultrasonic bath (Decon FS 100B, East Sussex).

3.2. Methods

3.2.1. Description of the study areas

Qualitative data obtained from field surveying and *Kocho* samples were collected from the farmers' field around Masha town of Sheka Zone. This is located in the Southern Nation Nationalities and People Regional State (SNNPRS) of Ethiopia. Masha town is situated at a distance of 951 km away from Hawassa (the capital city of the regional state in Southern Ethiopia). The geographical location of Masha town is given as follows: latitude and longitude of 7°44′N and 35°29′E, respectively, with elevation of 2223 m above sea level. The annual mean temperature ranges between 15.1–27.5^{o}C. The annual mean rainfall ranges between 1201–1800

mm and the soil type is red to brown. Other relevant information on the localities of the town could also be found in further details (http://www.snnprs.gov.et/Investment.pdf).

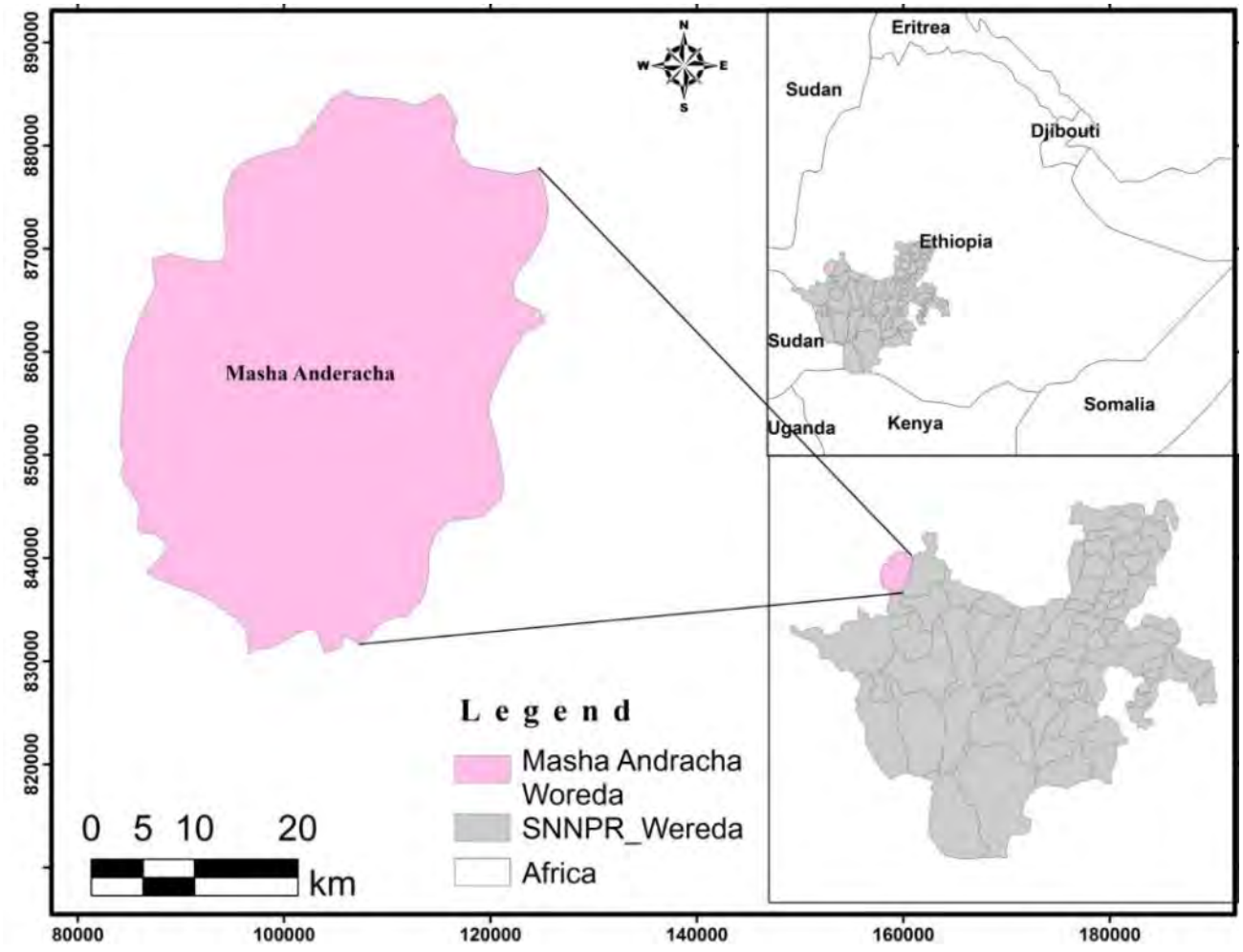


Figure 3.1 Research areas: Map of Masha and Anderacha districs

The study area was selected due to rich indigenous knowledge of the Shekacho society in *Enset* plantation, fermentation and consumption. In particular, the society has the tradition of using the stem of *Mandillo* plant to enhance fermentation processes of *Enset* in order to improve *Kocho* quality parameters. Furthermore, as high as 115 *Enset* local cultivars were also listed during the preliminary field survey of this study and they were locally classified into male and female types.

In this study, the female type *Enset* was selected for fermentation purposes considering the fast fermenting property of the cultivars.

3.2.2. Indigenous knowledge of the Shekacho society in *Enset* plantation management

3.2.2.1. Data collection methods

Data collection using questionnaire. Semi closed questionnaire composed of nine sections and ninety one questions was prepared in English language, validated with Sheka community living in Hawassa city; the capital of SNNPRS, and local society living in the research area with the assistance of translators when needed. Data were collected from two most *Enset* producing districts (Masha and Andracha). Fifty respondents were purposively and systematically selected among the knowledgeable women, farmers, agricultural experts, office employees and cultural leaders. Twenty one of the respondents were invited by the zone administrative officials. The remaining twenty nine respondents were selected based on the suggestions of the 21 respondents from different villages. The questionnaire was filled in by face to face discussion (Appendix D).

Data from different sources and field visits. Local annotations were also reviewed. Interviews were made with knowledgeable individuals among the 50 respondents, 12 farmers and the 2 women involved in fermentation process. To give evidence that *Enset* plantation has positive impacts on environmental protection and show the morphology of *Enset* plants, pictures of the research areas and available *Enset* plants were taken directly from the farms of twelve farmers. *Enset* local cultivars were listed by their local names throughout.

Data from the preliminary practical research work. Four matured female types (*Maache*) *Enset* plants were purchased from farmers nearby Masha town. Two knowledgeable women were selected and involved in the fermentation processes, who used their own local materials,

including wooden board (*matoo*), bamboo scraper (*maaroo*), wooden grater (*komixoo*), knife (*shikkoo*) and bucket (*dooloo*). Prior to fermentation, the site was cleared and pits (*gamoo*) were prepared. *Enset* leaves were also selected and exposed to sun to make it stronger and flexible. The knife was sharpened. Fermentation processes took place following the traditional methods by the local society and previous investigations (Hunduma and Ashenafi 2011; Gashe (1987a). All the leaves (*mato*), the interlocking leaf sheath (*dubo*) and dried leaf sheath (*kaakiro*) were removed from the *Enset* plants using knife. Parts of the pseudostem were scraped by *Maaroo* on *Matoo* slanted against a live *Enset.* The corm/*Amicho* was pulverized by *Komixoo.* The scraped and the pulverized biomasses were mixed and chopped by the knife. The chopped biomass samples were grouped into four equal portions, sealed carefully with the prepared *Enset* leaves and kept in the pits separately, for nine days. On the same day, the starter culture was prepared from different parts of *Enset* such as *shitoo* (pre-fermented lower part of midrib), *baqqoo* (inner soft part of pseudostem), *ooqqo* (midrib), *quuro* (squeezed liquid), *mundriiqqo* (the shoot) and *uuxo* (*Amicho*). The starter culture was prepared in duplicates, for both samples of fermentation with *Mandillo* (experimental) and without *Mandillo* (control) in all the four *kiisho-goonos* (bowel shaped structures made from underground corm). About 100 g of fresh *Mandillo* stem was added to evaluate its effect on product quality of *Kocho* and change in fermentation period of *Enset*. The bowels were kept intact to the ground. Both groups were sealed off from external air with leaf sheath and left for nine days.

At the end of the ninth day, all the partially fermented *Kocho* and the starter culture were taken out of the pit and *kiisho-goono,* respectively. The *Kocho* was kneaded and mixed with the starter cultures along with *Mandillo* (Kom) and without *Mandillo* (Koki). After mixing, the contents were buried in the pits again for another five days. On the fifth day, the *Kocho* samples were

removed from the *gamoo* and pancake like bread were prepared. Sensory attributes (color, taste, odor, softness, texture and maturity) of the starter culture, *Kocho* dough and the baked pancake like breads were evaluated by selecting 30 local consumers from Masha town using a five points hedonic scale (Abebe *et al.*, 2006).

3.2.2.2. Plant material collection and preparation

Fresh *Mandillo* samples were collected on November 2011 and 2015. Sample preparation was done according to the reported methods by Shakeri *et al*. (2012) and Shabir *et al*. (2011). The plant materials were identified at the Department of Plant Biology and Biodiversity Management, Addis Ababa University, Ethiopia. To evaluate the phytochemical nature of *Mandillo,* the plant samples were grouped into stem, leaves and aerial parts. The three parts were dried at room temperature under shade for three days (Zohra *et al*., 2012). The dried samples were ground to fine powder using electric grinder to increase its surface area. Moisture contents of fresh and dried samples were analyzed by drying oven at 105^{o}C, until constant weight was obtained. The stock samples were kept in air tight polyethene bag at 4^{o}C for extraction (Sibanda, 2008).

3.2.3. Evaluation of phytochemical, antioxidant and antimicrobial activities of *Mandillo*

3.2.3.1. Preparation of extracts

The plant materials were extracted based on the methods reported by Sultana (2009) and Eom *et al*. (2008). Air dried powder of *Mandillo* leaves, stem and aerial part were extracted three times by socking 100 g sample in 1,000 mL (Taiwe, 2012) of 70% ethanol (Addai *et al.*, 2013), 95% methanol, ethyl acetate, acetone and distilled water (DW) on orbital shaker at 130 rpm heated to 40°C for 24 h. The extracts were filtered using Whatman No 1 filter paper. The extracts were

then concentrated by rotary evaporator at 40°C. The resulting masses of crude extracts were weighed, labeled and kept at 0^{o}C in brown bottles for further study.

3.2.3.2. Phytochemical screening

Phytochemical screening was carried out according to the methods reported by Uma and Sekar (2014), Tiwari *et al*. (2011), Akinyemi (2005) and Sofowora (1993). Extracts were separately dissolved in dilute hydrochloric acid and filtered. The presence of alkaloids was evaluated by Wagner's test. The filtrates were treated with iodine in potassium iodide [Wagner's reagent (2 g I_2 and 6 g KI in distilled water; *w/w/v*)] and further treated with few drops of 10% lead acetate solution. The formation of yellow color precipitates confirms the presence of flavonoids. A 10 mg of each extract was dissolved in 1 mL of chloroform. A 1 mL of acetic anhydride was added following the addition of 2 mL concentrated H_2SO_4. Formation of reddish violet color indicates the presence of terpenoids. A 0.5 g of each of the extracts was shaken with 2 mL water. Formation of foam which persisted for 10 min, indicates the presence of saponins. A 1% gelatin solution containing sodium chloride was added to each of the extracts. The formation of white precipitate indicates the presence of tannins. A 5 mL of each of the extracts solution was hydrolyzed with concentrated H_2SO_4 and extracted with benzene. A 1 mL of dilute ammonia was added to each extracts. Rose to pink coloration confirms the presence of anthraquinones. A 1 mL of each of the extracts was dissolved in 10 mL chloroform with equal volume of concentrated sulphuric acid being added by the sides of the test tube. The upper layer turns red and the acid layer to yellow with green fluorescence indicating the presence of steroids.

3.2.3.3. Determination of total phenolic contents

Total phenolic contents were determined according to the Folin-Ciocalteu's procedure, following the method used by Alimpić (2014). The stock solutions of *Mandillo* extracts were diluted to 0.1 mg/mL in methanol and 100 µL was transferred into test tubes in triplicates. A 1 mL of Folin-Ciocalteu's reagent (diluted 1:10) was dissolved in deionized water and added to each of the test tube. A 1 mL of sodium carbonate (7.5%) reagent was added to each mixture. The tubes were vortexed and allowed to stand at 25^{o}C for 90 min in incubator. Absorbance was measured at λ_{max} 765 nm by a spectrophotometer. The total phenolic content was determined using a standard curve of gallic acid concentrations of 0.001–1.00 mg/mL in methanol and the results were calculated using(. The values were expressed as Gallic acid equivalents in milligrams per gram of dry material (mg GAE/g).

Where, y = absorbance the extract, x = concentration of the extract, and R = regression coefficient

3.2.3.4. Determination of total flavonoid contents of *Mandillo*

Total flavonoid contents were determined using the method reported by Sultana (2012). A 1 mg/mL plant extract from stock solutions was prepared. A 2 mL of 2% aluminum chloride with the same volume of *Mandillo* extracts was made to each test tube. A 2 mL of each sample solution without aluminum chloride was prepared in 2 mL of methanol and used as a blank. The absorbance reading of each sample was taken at λ_{max} 415 nm after 30 min incubation at room temperature against the blank sample by a spectrophotometer. The total flavonoid contents were determined using the standard curve of quercetin concentrations of 0.001–40 µg/mL with 2 mL of 2% aluminum chloride in triplicate. The values

were expressed as milligram of quercetin equivalent per gram (mg QE/g) of dry *Mandillo* samples.

3.2.3.5. Evaluation of antioxidant capacity of *Mandillo* by DPPH assay

Antioxidant scavenging capacity was analyzed according to the procedure (Oloyede and Ogunlade 2013). Each extract was mixed with methanol to prepare a solution of 1 mg/mL. Solutions of 5 µL, 10 µL, 20 µL, 35 µL, 50 µL and 60 µL of the extracts were taken in test tubes in triplicates. Serial dilutions of the above solutions were made by adding 995 µL, 990 µL, 980 µL, 965 µL, 950 µL and 940 µL of methanol to the final volume of 1 mL, respectively. The final concentrations were calculated as 0.005 mg/mL, 0.01 mg/mL, 0.02 mg/mL, 0.035 mg/mL, 0.05 mg/mL and 0.06 mg/mL, respectively. A 2 mL of freshly prepared 2, 2-diphenyl-1-picryl hydrazyl (DPPH) solution (0.06% w/v) in methanol was added to each test tube containing *Mandillo* extracts. The reaction mixture and 2 mL control (DPPH in methanol) was vortexed and left to stand at room temperature in dark place for 30 min. The absorbance of the resulting solution was measured at λ_{max} 517 nm by a spectrophotometer. Ascorbic acid was used as a reference standard. The control was prepared by adding 1,000 µL methanol to 2 mL of DPPH solution. The DPPH radical scavenging capacity of the plant extracts was calculated as percent of DPPH scavenging power. [illegible] The DPPH scavenging potentials of the extracts were measured as IC_{50}, which is the concentration of the sample required to inhibit oxidation at 50% of the concentration of DPPH.

Where, ADPPH is absorbance of 2, 2-diphenyl-1-picryl hydrazyl (DPPH) and Aextract is absorbance of the extract.

3.2.3.6. Evaluation of DNA-protective activity of *Mandillo*

DNA protecting activity of *Mandillo* stem extract with 70% ethanol was evaluated against DNA damaging chemicals according to the method used by Kim *et al*. (2012) as cited by Rafiquzzamana (2013). Hydroxyl radicals were generated by the mixture of 30 µL of ascorbic acid (10 mM final concentration) and 1 µL of copper sulfate (II) (1 mM final concentration). Bacteriophage λ DNA (40 µL, 0.1 µg/mL) was exposed to the solution in the absence as well as in the presence of *Mandillo* (100 µL, 1 mg/mL). The mixture was then incubated at 37^{o}C for 1 h after which the samples were loaded onto a 1% agarose gel and fragments were separated by Agarose gel electrophoresis (Rafiquzzamana, 2013). The result was compared with the control group (λ DNA alone)

3.2.3.7. Analysis of antimicrobial activity of *Mandillo*

Antimicrobial activity of *Mandillo* was evaluated according to the literature information; Lee *et al*. (2014). The microorganisms used were *Escherichia coli* (KCTC 1682), *Enterococcus faecalis* (KCTC 3206), *Staphylococcus aureus* (KCTC 3881), *Lactobacillus plantarum* (M.B.P. 06) and *Candida utilizes* (KCCM 11355). The microorganisms were obtained from the Korean Collection for Type Culture Centers (KCTC; Daejeon, Korea), the Korea Culture Center of Microorganisms (KCCM; Seoul, Korea) and Department of Food Science and Technology, College of Fisheries Sciences, Pukyong National University (Busan, South Korea). The bacteria and yeast strains collected were cultured in Muller Hinton broth (MHB; Difco, Sparks, Maryland, USA) (Rimek *et al*., 2008) at 37^{o}C for 12 h. Each inoculum was adjusted to 0.5 McFarland standards, equivalent to 1×10^{8} CFU/mL (Wiegand, 2008; EUCAST, 2000), which was used to test antimicrobial activities. Determination of the minimum inhibitory concentration

(MIC) and minimum bactericidal/fungicidal concentration (MB/FC) of *Mandillo* were estimated by broth dilution and agar plate count techniques, respectively. The culture tubes were inoculated with respective cultures of about 10^5 CFU/mL. The stock solution of *Mandillo* stem extract (10 mg/mL) was diluted by 2-fold serial dilution method. Seven different concentrations of *Mandillo* extracts were tested in 96-well micro plates (15.63 µg/mL, 31.25 µg/mL, 62.50 µg/mL, 125.00 µg/mL, 250.00 µg/mL, 500.00 µg/mL and 1,000.00 µg/mL) (Lee *et al*., 2014; Wiegand, 2008). The inocula were incubated at 37°C for 24 h. The optical density (OD) of each culture in well inocula was determined at λ_{max} 600 nm at 12 and 24 h, using ELISA Micro Plate Reader (Nshimiyumukiza, 2015). The lowest concentration of plant extract that inhibits and did not permit any visible growth of the inoculated test organism in broth culture was regarded as the MIC in all cases. Growth inhibition is defined as an OD of < 0.08 (Thongson, 2005). After culturing the test organisms separately in MHB, containing various concentrations of the plant extracts, the broth was inoculated onto freshly prepared MRS agar, MHA and PDA plates to assay for the bactericidal/fungicidal effect. The cultures were incubated at 37°C from 24 h to 5 days. The lowest concentration of plant extract that does not yield any colony growth (99.9%) on the solid medium after the incubation period (48 h to 5 days) was regarded as MB/FC (Thongson, 2005).

3.2.3.8. Cytotoxicity evaluation of *Mandillo* stem by MTT assay

Crude extraction. Powdered *Mandillo* stem weighing 100 g was extracted with 1000 mL of 70% ethanol on orbital shaker at 130 rpm for 24 h heated to 40°C. The extracts were then concentrated and dried under reduced pressure using rotary evaporator at 40°C. The resulting masses of the crude extracts were weighed, labeled and kept at 0°C for further study.

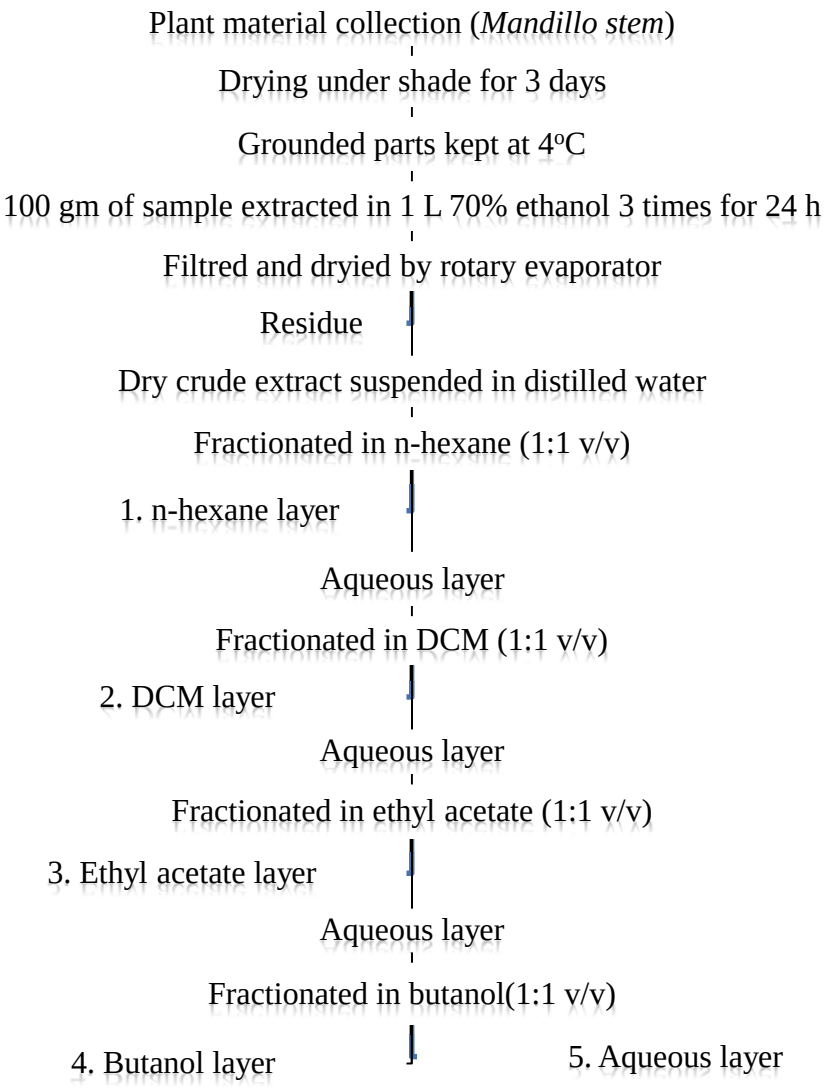


Figure 3.2: Fractionation of *Mandillo* stem crude extract in different solvents.

Fractionation. The dry crude stem extract was suspended in distilled water and successfully partitioned with n-hexane, dichloromethane (DCM), ethyl acetate and butanol (Figure 3.1) (Eom *et al*., 2008). Fractionates were dried under reduced pressure using rotary evaporator at 40°C and kept at 0^{o}C till used for cytotoxicity evaluation.

Cytotoxicity evaluation. The cytotoxicity of the stem extracts of *Mandillo* was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT); a method based on MTT

assay (Saleem *et al*., 2014; Nemati *et al*., 2013; Choi *et al.*, 2012; Sini *et al*., 2012; Mat *et al.*, 2011; Carneiro *et al.*, 2009).

Incubation of the raw cells. Raw Cell 264.7 obtained from the American Type Culture Collection (ATCC; Manassas, USA), was grown to confluence in Dulbecco's Modified Eagle medium; DMEM (Gibco BRL, NY, USA) with 1% penicillin (100 U/mL and glutamine 2 mM; Gibco BRL), streptomycin (100 µg/mL; Gibco BRL) and heat inactivated 10% fetal bovine serum (FBS; Gibco BRL) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air at 37^{o}C in flasks. The medium containing the Raw Cells 264.7, at a density of 10^5 cells/mL was cultured with 100 µL in a 96-well plate; incubated under 5% CO_2 and 95% air at 37^{o}C for 24 h**.**

Treatment of the raw cell with *Mandillo* stem extract. The Raw Cells 264.7 were treated with different concentrations of *Mandillo* extracts of (2, 4, 10, 20, 50, 100 and 200 µg/mL)**.** Dilution of the stock solutions was made in culture medium yielding final extracts concentrations with a final dimethyl sulfoxide (DMSO) concentration of 0.1%. Control Raw Cells 264.7 were incubated in culture medium only (without the plant extract). All concentrations of *Mandillo* extracts were in triplicates on the same cell batch. After 24 h of incubation, 100 µL of MTT solution was added to each well and the plate was incubated for another 4 h at 37°C. The formation of insoluble purple formazan from yellowish MTT by enzymatic reduction was dissolved in 100 µL DMSO after removal of MTT supernatant.

Measurement of the optical density of formazan. Optical density of the solution was measured at 540 nm using ELISA micro plate reader. The optical density of formazan formed by untreated Raw Cells 264.7 was taken as the measure of 100% cell viability. The IC_{50} is defined as the concentration of the extract that produces a 50% decrease in cell viability, relative to the negative

control (wells were exposed to the solvent without any extract) was considered as cut off point for toxicity. The percentage growth inhibition was calculated as follows:

% cell inhibition = $100 - [(A_t-A_b)/(A_c - A_b)] \times 100$…….(1)

where, A_t, absorbance value of test compound; A_b, Absorbance value of blank and A_c, Absorbance value of control.

3.2.4. Evaluation of the effects of *Mandillo* during *Enset* fermentation

3.2.4.1. *Enset* fermentation processes and *Kocho* sample collection techniques

Kocho samples were collected from *Enset* fermentation pits and jars. Fermentation processes were following the traditional knowledge of Shekacho society and the standard methods reported by Karssa *et al.* (2014), Hunduma and Ashenafi (2011) and Gashe (1987a) as described above.

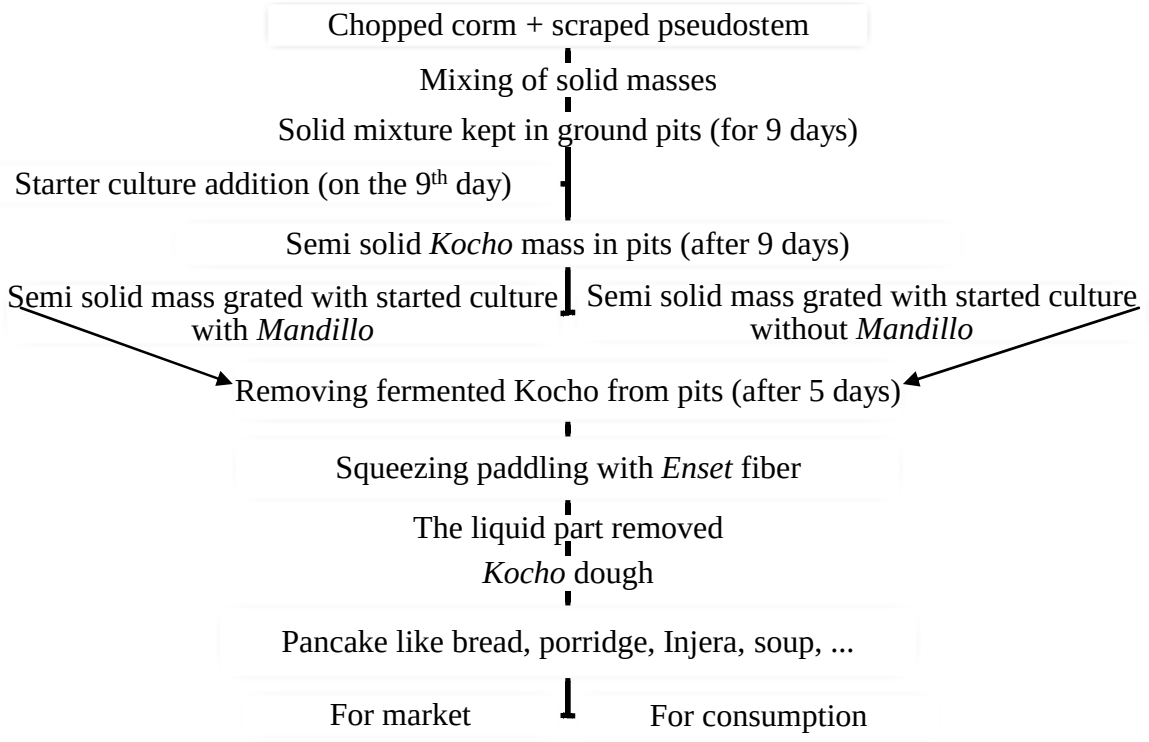


Figure 3.3: Flow diagram of *Kocho* fermentations

Fermentation of samples were classified into initiation period *Kocho* (Ko*)* fermented in the pits (days 0–9), control *Kocho* (Koki) and experimental *Kocho* (Kom) samples fermented in the jars (days 9–45); sealed with polyethylene bags. Fermentation of Ko samples were initiated in Masha town, whereas, Koki and Kom took place at the Food Microbiology Laboratory, Center for Food Science and Nutrition, Addis Ababa University, Ethiopia. The 9^{th} day partially fermented Kocho (Ko) was used as initial sample for Koki and Kom groups and the starter cultures were added to the Ko sample. All the three sample groups were fermented in triplicate. The initiation period *Kocho* (Ko) samples were fermented without starter culture. The starter culture of the control *Kocho* (Koki) samples were prepared without *Mandillo* stem. *Mandillo* stem was added to the starter culture of the experimental *Kocho* (Kom) samples as one part of the starter culture ingredients. The fermentation processes carried out twice at room temperature (14–18^{o}C) and their average results were used for data analysis. *Kocho* samples for evaluations of pH value, titratable acidity, lactic acid and acetic acid concentrations, microbial dynamics and microbial diversity were aseptically collected from the fermentation pits (days 0–9) and jars (days 11, 15, 21, 29, 37 and 45). The samples were kept at 2–4^{o}C for further analysis.

3.2.4.2. Evaluation of pH values, titratable acidity, lactic acid and acetic acid contents

3.2.4.2.1. Measurement of pH values

The pH value was determined using a pH meter with a glass electrode. A 10 g of each sample of *Kocho* was dissolved in 90 mL distilled water. The pH meter was calibrated prior to each reading with standard buffers of pH 4.00, 7.00 and 10.00 (Oyedeji *et al*., 2013).

3.2.4.2.2. Titratable acidity analysis

Titratable acidity was determined by using the method of Oyedeji *et al*. (2013). A 10 g of each sample of *Kocho* was dissolved in 90 mL distilled water. The suspension was titrated against 0.1 N NaOH to phenolphthalein end point. The relative lactic acid equivalent is the amount of NaOH consumed in milliliter. Each milliliter of 1 N NaOH is equivalent to 10.008 mg of lactic acid.

3.2.4.2.3. Lactic acid and acetic acid analysis

Lactic acid and acetic acid contents were extracted as described by Sanchez-Machado (2008). A 10 g of each sample was dissolved in 90 mL deionized water. The content was homogenized and heated in water bath at 65°C for 5 min to prevent further microbial activities. The samples were vortexed for 1 min and neutralized to pH 7 by adding 0.1 N NaOH using pH meter. The tubes were placed ultrasonic bath for 5 min to facilitate the extraction of organic acids. The homogenates were centrifuged at 4,000 rpm for 10 min using low speed centrifuge. The acid containing supernatants were filtered through cellulose acetate membrane with pore size of 0.45 µm and 1.5 mL of each filtrate was transferred to vials in triplicates. Chromatographic analyses were performed using HPLC instrument equipped with quaternary pump, vacuum degasser, auto sampler and diode array detector (DAD). Chromatographic separations were performed on analytical column. Data acquisition and processing were accomplished with LC chemstation software. Chromatographic separations were carried out using mobile phase of 0.005 N H_2SO_4. Analysis was performed with the column temperature 14^{o}C, detection wavelength of λ_{max} 210 nm with UV detector and flow rate of 0.6 mL/min, in isocratic mode. Standard curves were prepared from pure and known concentration of a mixture of acetic and lactic acids (0.10–1.00 mg/L). A 1.00 mg/L of single standards' solutions were run to know the peak time of the acids. Then, 10

μL samples were injected to the HPLC automatically and eluted for 10 min run time and 1 min post-time. Finally, peak area was utilized as instrumental response.

3.2.4.3. Enumeration of microbes

Microbial counts were conducted according to the procedure described by Cain *et al.* (2013). A 10 g of Ko, Koki and Kom samples were aseptically removed from the pits and Jars. The samples were homogenized with 90 mL sterile distilled water using orbital shaker at 250 rpm for 30 min (Hunduma and Ashenafi, 2011; Abegaz, 2007; Pulido *et al*. 2005; Idris, 2001; Gashe, 1987a). A ten-fold serial dilution was made and from appropriate dilution factor, 0.1 mL of each aliquot was aseptically taken and spread-plated on respective pre-dried agar media such as de Man, Rogosa, and Sharpe (MRS) agar, Potato Dextrose agar (PDA) amended with 50 mg/L of chloramphenicol, Plate Count agar (PCA), Violet Red Bile agar (VRBA) and Mannitol Salt agar (MSA) for lactic acid bacteria (LAB), yeast, aerobic mesophilic bacteria, total coliforms and staphylococci counts, respectively. Aerobic spore formers were counted on Plate Count (PC) agar after appropriate dilution was heat-treated at 80^{o}C for 10 min in water bath. All inoculated plates except for the yeast were incubated at 30^{o}C for 24–48 h. LAB inoculated plates were incubated under anaerobic conditions in gaspak jar. Yeast inoculated plates were incubated at 28°C for 24–72 h. All plates with 30–300 colonies were counted.

3.2.4.4. Isolation of LAB and yeast strains

Ten to twenty presumptive colonies of LAB and yeast were randomly picked from MRS agar and PDA, respectively, and isolated. The isolates were purified on their respective culture media by repeatedly streaking on appropriate agar media. Pure isolates were maintained on agar slants and stored at 2–4^{o}C for the subsequent activities.

3.2.4.5. Physico-chemical characterization of the LAB and yeast isolates

Isolates from the presumptive plates of LAB and yeast were subjected to different morphological, and biochemical tests based on the methods of Ni (2015), Karssa *et al*. (2013), Nikita and Hemangi (2012), Abd El Gawad (2010), Abegaz (2007) and Gashe (1987a).

The yeast isolates were physically identified from molds by the use of microscope (Musgrove *et al.,* 2008). Yeast isolates were further evaluated by spore staining (Cain *et al.,* 2013). The stained slide was viewed under oil immersion (magnification of 1000×) with a light microscope. The LAB isolates were evaluated for shape, catalase test, Gram stain, KOH string test, spore formation and motility test.

3.2.4.5.1. Catalase test

To check the production of enzyme catalase, a clean microscopic slide was used. A drop of 3% H_2O_2 (*v/v)* was put on the microscopic slide aseptically. A loopful of bacterial culture was taken and mixed with the H_2O_2 solution on the slide and the absence of bubble production was observed.

3.2.4.5.2. Gram’s staining

A 24 h old LAB was used for Gram's staining. The colonies were spread over a thin layer across a microscope slide containing a drop of water and allowed to air dry. The smear was fixed and covered with crystal violet for 1 min. After rinsing the slide under gently running tap water, the smear was then flooded with Gram’s iodine solution for 1 min and washed gently with organic solvent (95% ethanol). After rinsing the slide, it counter stained with Safranin for 1 min. The stain was gently rinsed off by tilting the slide under a lightly and regular running tap water until the water runs clear. The stain was blot drying with clean cloth. Gram-positive cells were

appeared purple and Gram-negatives red after proper staining under oil immersion objective (magnification 1000X) microscope.

3.2.4.5.3. KOH string test

KOH string test was carried out using a drop of 3% potassium hydroxide (*w/v*) on a glass slide. A visible loopful of cells from a single, well-isolated colony was mixed into the drop. The colony which didn't turn viscous was considered as gram-positive.

3.2.4.5.4. Endospores test

Microbial smears were made on microscopic slides and heat fixed. Then slides were placed over the steaming water bath and Malachite Green (primary stain) was applied for 5 min. The slide was removed from the water bath and cooled for 5 min. Thereafter, the slides rinsed with water until water run clear. The slides were flooded with the counter stain (safranin) for 20 s. and rinsed with water. The slides were blot dried and observed under oil immersion lens with a light microscope (magnification 1000X).

3.2.4.5.5. Motility test

MRS agar was used for testing if the bacteria were motile or non-motile through stab inoculation into semisolid agar medium in a test tube and incubated from 24 to 48 h.

3.2.4.5.6. Physiological tests

The isolates were also subjected to physiological and biochemical tests to characterize to the genus level. Growth at various temperatures (10^{o}C, 15^{o}C and 45^{o}C), tolerance of different concentrations of NaCl (4.0%, 6.5% and 8% (*w/v*)) and pH (3.9, 4.4, 6.5 and 9.6) were conducted.

3.2.4.5.7. Sugar fermentation test

To identify hetero and homo-fermentative activities, glucose fermentation test in MRS broth using inverted Durham's tubes was evaluated (Devi, 2013; Azadnia and Nazer, 2009). A 100 mL of nutrient broth solution was prepared at different time in conical flask to which 1 mL phenol red was added. This medium was autoclaved at 121°C for 15 min and cooled at room temperature. A syringe filter sterilized solution of 1% glucose was prepared under aseptic conditions. In all sterilized test tube, 5 mL of the broth and 100 µL of the glucose solution were mixed and labeled. The test tubes were kept at room temperature for 24 h to check the contamination. After 24 h, all the test tubes were inoculated with freshly grown isolates and incubated at 37°C for 48 h. In cases of homofermentation, there were production of acid along with the change in color of the medium from red to yellow, and in heterofermentation, gas in Durham's tubes and acid productions along with the change in color from brown to yellowish.

3.2.4.6. Genomic DNA extraction and PCR amplification of 16S rRNA and 18S rRNA genes

3.2.4.6.1. Selection of isolates for molecular analysis

Presumptive LAB and yeast representative isolates were selected from the total isolates for 16S rRNA and 18S rRNA genes sequencing analysis, respectively (Abd El Gawad *et al.,* 2010). Most dominant isolates were randomly picked proportional to represent the groups and all rare strains were included.

3.2.4.6.2. DNA extraction from LAB isolates

Overnight grown LAB yeast isolates were used for DNA extraction. A 100 µL lysine buffer was first added to each 1.5 mL capacity Eppendorf tube. A 100 mg/mL of 2.0 µL lysozyme and 20 mg/mL of 2.0 µL Proteinase K were added. A loopful of overnight grown LAB colonies was added into the buffer solution and vortexed. The solution was heated in water bath at 65^{o}C for 30

min and then boiled at 100^{o}C for 10 min. It was centrifuged at 13,000 rpm for 10 min. The LAB extract was kept at -20^{o}C for PCR processing

3.2.4.6.3. DNA extraction from yeast isolates

Overnight grown yeast isolates were used for DNA extraction. A 100 µL lysine buffer was added to each 1.5 mL capacity Eppendorf tube. A 2.5 unit/mL of 1.0 µL lyticase and 20 mg/mL of 2.0 µL of Proteinase K were added. A loopful of overnight grown yeast colonies was added into the buffer solution and vortexed. The solutions were heated in water bath at 65^{o}C for 30 min and then boiled at 100^{o}C for 10 min. It was centrifuged at 13,000 rpm for 10 min. Yeast extract was kept at -20^{o}C for PCR processing.

3.2.4.6.4. Polymerase chain reaction (PCR) processes of DNA and amplicon sequencing of LAB and yeast

A 3.0 µL of genomic DNA of LAB and yeast isolates were used for PCR processing. PCR was run using SolGent's PCR series (Korea). PCR ingredients were included 3.0 µL of DNA sample, 1.0 µL of 10 pmole of each forward primer 27F (5'AGA GTT TGA TCC TGG CTC AG3') and reverse primer 1492R (5'GGT TAC CTT GTT ACG ACT T3') for LAB and forward primer ITS1 (5'TCC GTA GGT GAA CCT GCG G3') and reverse primer ITS4 (5'TCC TCC GCT TAT TGA TAT GC3') for yeast (Sacchi *et al*., 2002), 12.5 µL of SolgTM 2X EF-Taq PCR Smart Mix 1 (Cat. No. SEF01-M50h). Sterile distilled water filled to 25 µL of total PCR volume. A PCR was performed under the following conditions: preheating was done at 95^{o}C for 15 min for 1 cycle, denaturation at 95^{o}C for 20 s, annealing at 55^{o}C for 40 s and extension at 72^{o}C for 15 min run for 35 cycles. The 16S/18S rRNA gene was amplified with the universal bacterial primer

pairs above (Weisburg*et al*., 1991) and the purified PCR products (amplicon) were sequenced according to the protocol of the company, SolGent Co. Ltd (Daejeon, Korea)

3.2.4.7. Phylogenetic relationships of taxa evaluation of LAB and yeast

LAB and yeast 16S and 18S rRNA, respectively gene sequences were first clustered by BLAST comparison (Clarridge, 2004) and sorting identical strains from each phylogenic tree of 111 LAB and 50 yeast isolates. Similarities in nucleotide or differences in base pairs (bp) were checked manually using Molecular Evolutionary Genetics Analysis version 6.0 (MEGA 6) (Tamura, 2013). As a fourth method of clustering, Bayesian rRNA Classifier Version 2.10 software at confidence threshold of 80% (Appendix B) (Ntushelo, 2013; Wang *et al*., 2007) was used. Finally, the sequences were further identified by reconstructing phylogenic tree selecting representative strain from each cluster with reference sequences and relevant out group strains. The evolutionary history of LAB and yeast were inferred using the Neighbour Joining method (Saitou and Nei, 1987). The confidence probability estimated using the bootstrap test (1000 replicates) (Dopazo, 1994; Rzhetsky and Nei, 1992; Felsenstein, 1985). There were a total of 1331 LAB and 367 yeast positions in the final dataset. Phylogenetic trees were constructed using MEGA 6 (Tamura, 2013).

3.2.4.8. Evaluation of food *(Kocho)* composition

3.2.4.8.1. *Kocho* sample collection techniques

Kocho samples for food compositions and sensory analyses were aseptically collected from *Enset* fermentation jars on day 37 and extended fermentation days of 75 and 90. The samples were kept at 2–4^{o}C and used for the analysis.

3.2.4.8.2. *Kocho* composition analysis

In both fermentation times, Koki and Kom samples of days 37, 75 and 90 were analyzed for proximate composition (ash, moisture, fiber, protein, fat, and carbohydrate contents), mineral contents (Ca, Fe, Zn and Cu), water holding capacity, anti-nutritional factors (phytic acid and tannins) and sensory attributes.

3.2.4.8.3. Analysis of proximate composition

The moisture contents, crude fiber, ash, crude protein, total fat and carbohydrate were evaluated according to the standard methods of the Association of Official Analytical Chemists (AOAC, 2000) and the methods used by Aberoumand (2011), Gashe (1987a) and Gashe (1987b).

3.2.4.8.4. Determination of moisture contents

Moisture content of the *Kocho* samples were determined according to AOAC (2000), briefed as follows: First, moisture of the dishes was removed by heating in a drying oven at 130^{o}C for 1 h (Hussain, *et al*., 2010). The dishes were placed in adesiccator for 30 min to cool and then weighed (M_1). About 5 g *Kocho* sample was transferred into each dried dish and weighed (M_2). The contents were dried at 105^{o}C in the drying oven for 6 h. After successive heating to a constant weight (M_3), the moisture contents were calculated using the following equation:

$$\text{Moisture content (\%)} = \frac{M_2 - M_3}{M_2 - M_1} \times 100 \quad \ldots\ldots\ldots (2)$$

where M_1: weight of dry dish, M_2: weight of fresh sample and dish and M_3: weight of dry sample and dish.

3.2.4.8.5. Determination of ash contents

Ash content determination was performed following the standard method of the AOAC (2000). Accordingly, porcelain crucibles were dried in an oven at 105^{o}C for 30 min and cooled in a desiccator for 30 min (M_1). Then, 2 g *Kocho* sample was transferred to each of the dried crucible and weighed (M_2). The entire contents were then ignited in a muffle furnace (Aberoumand, 2011) at 550^{o}C for 1 h to obtain ashes. Thereafter, the crucibles were taken out from the furnace and cooled. This was followed by moistening of the resulting ashes with few drops of deionized water, which was evaporated on a hot plate. The contents in the crucible were ignited again for 30 min at 550^{o}C and cooled. Few drops of deionized water and 5 drops of concentrated HNO_3 were added and again evaporated on a hot plate. The contents were further ignited at 550^{o}C for 30 min and the crucibles were finally cooled in a desiccator for 45 min and weighed (M_3). The ash contents were calculated utilizing the following equation:

$$\text{Ash content} = \frac{M_3 - M_1}{M_2 - M_1} \times 100 \quad \ldots\ldots\ldots (3)$$

where: M_1 - weight of dry crucible, M_2 -weight of fresh sample and crucible and M_3 -weight of dry sample and crucible.

3.2.4.8.6. Determination of total nitrogen (protein)

Sample digestion. Crude protein contents of Koki and Kom were determined by Kjeldahl method (AOAC, 2000). About 2 g *Kocho* sample was weighed in a tecator tube placed in tecator rack. A 6 mL acid mixture (concentrated ortho-phosphoric acid and sulphuric acid (1:20 *v/v*) was added and the content was instantly mixed. Then, 3.5 mL of 30% hydrogen peroxide was added to each sample tube, drop by drop, in a fume hood, which resulted in a vigorous reaction to take

place. At the end of the reaction, the tubes were shaken manually for few min and returned to the racks. This was followed by addition of 3 g mixture of $CuSO_4$ and K_2SO_4 (1:15 *w/w*) to each tube for further analysis of the subsequent reaction. Afterwards, the tubes were allowed to stand for 15 min before digestion. The tubes containing the samples were put into the rack. The rack containing the tubes were placed into the digesters and first heated to 370^{o}C in the fume hood, equipped with exhaust manifold positioned on the top of the tubes. Then, the digestion was allowed to take place for 3 h, until clear solution appeared in the fume hood. Finally, the samples were kept and cooled in the fume hood.

Distillation and titration of the digested samples. The blank and the sample have undergone steam distillation in Kjeltec analyzer unit (Hussain *et al*., 2010) with 40% sodium hydroxide (*w/v).* It was observed that as a result of the entire reactions, all nitrogenous containing compounds were converted into ammonium, which was confirmed by the release of ammonia. Finally, the ammonia gas released was trapped by 1% boric acid and titrated against 0.1 N HCl. (Boric acid solution used as indicator was prepared from 75 mg of bromocresol green and 50 mg of methyl red in 100 mL of 99% ethanol). Nitrogen content of the *Kocho* samples were then calculated using equation given below:

$$\% \text{ Nitrogen} = \frac{(T_s - T_b) \text{ x Normality of acid x meq. of } N_2}{\text{Weight of sample (in gram)}} \text{ X } 100 \text{ ……. (4)}$$

Where, T_s = Titration volume of the sample (ml), T_b = Titration volume of the blank (mL), meq. of N_2 = 0.014 and % protein = % Nitrogen × 6.25. % protein = % N x 6.25

3.2.4.8.7. Determination of total fiber

The total fiber content of the *Kocho* sample was determined using the standard method of AOAC (2000). Accordingly, about 2 g of the *Kocho* samples were weighed and added into each of the 600 mL beaker and recorded as M_1. The samples were then digested with 200 mL of 1.25% of sulphuric acid (*v/v*) for 30 min and filtered. The resulting contents were immediately treated with 20 mL of 28% KOH (*w/v*) and boiled gently for further 30 min; while stirring occasionally. The bottom of a sintered glass crucible was covered with 10 mm sand. The layer of sand was wetted with little distilled water. The solution poured into sintered glass crucible washing the beaker several times. Residue in crucible was washed with hot, distilled water and filtered twice. Thereafter, the residue was washed with 1% H_2SO_4 (*v/v*) and filtered. The residue further washed with hot distilled water and filtered. Washing of the residue was done with 1% NaOH (*w/v*) and filtered. It was washed again with hot distilled water and filtered and finally washed with 1% H_2SO_4 and then filtered. The residue was washed twice more with hot distilled water and filtered. The sintered glass crucible placed under a vacuum pump at 105^{o}C for 2 h. The dried residue was placed in the oven and dried at 105^{o}C for 2 h. The samples were cooled in the desiccator for 30 min and weighed (M_2). The dried samples were heated at 550^{o}C to make ash. It was cooled in a desiccator and weighted (M_3). The crude fiber was calculated using the following equation (Hussain *et al*., 2010).

$$\text{Crude fiber} = \frac{M_2 - M_3}{M_1} \times 100 \quad \ldots\ldots\ldots (5)$$

where: M_1, weight of sample; M_2, weight of fiber ash and crucible and M_3, weight of ash and crucible

3.2.4.8.8. Determination of total fat

Total fat was determined using a standard method of AOAC (2000). Accordingly, about 2 g of the *Kocho* samples was transferred to each of the 100 mL extraction flask. The fat content of *Kocho* samples were determined by Soxhlet extraction method using 70 mL diethyl ether as extraction solvent. The ethyl ether was evaporated from the extraction flask. The amount of fat in each *Kocho* sample was calculated from the difference in weight of the extraction flask before (M_1) and after extraction (M_2) and calculated using the following equation: (Hussain *et al*., 2010).

□□ □□ – □□ □□□□ □ □□□□ □□□□□……. (6)

M_f: weight of crude fat content in *Kocho* samples; M_1, weight of extraction flask before extraction and M_2, weight of extraction flask after extraction

3.2.4.8.9. Determination of carbohydrate contents

Total carbohydrate content was determined from the difference, in weights of the various parameters determined within the proximate analysis as indicated in the following relation (Aberoumand, 2011).

□□□□□□□□□□□□□□□□□ □□□□□□□□□ □ □ □□□ – [moisture (%) − protein content (%) − crude fat (%) – ash (%) – fiber (%)]……. (7)

3.2.4.8.10. Evaluation of the mineral contents

Mineral contents of the *Kocho* sample were determined following the methods reported by Hussain *et al*. (2010). In order to evaluate the amount of trace metals such as calcium, copper,

iron and zinc, dry *Kocho* samples were ignited to obtain ash. First, six porcelain crucibles were washed with 6 N HCl while the glass wares were washed with 10% nitric acid (*v/v*). The crucibles were placed in an oven at 105^{o}C for 30 min and then cooled in the dessicators for another 30 min.

Sample ashing. About 2.5 g of *Kocho* sample was placed in each crucible. The samples were charred in a hot plate in fume hood, by successively increasing the temperature. The samples were made ash in a muffle furnace at 550^{o}C for 1 h. The crucibles were then taken out of the furnace and cooled. The resulting samples were moistened with few drops of deionized water and evaporated on a hot plate. Afterwards, the samples were ignited for the second time at 550^{o}C for 30 min and cooled. Drops of deionized water and 5 drops of concentrated HNO_3 were added to the cooled sample and evaporated on a hot plate. The water and acid treated and dried samples were further ignited, for the third time, for 30 min at the same temperature as described in this section. The crucibles were finally cooled in a desiccator for 45 min and weighed.

Sample homogenization. The ash obtained in the previous processes was treated with 10 mL of 6 N HCl to wet. It was completely and carefully taken to dryness by heating on a hot plate at low temperature. Then, 15 mL of 3 N HCl was added and the crucible was heated again on the hot plate until the solution was boiled. Then, the resulting solution was cooled and filtered through filter paper into a graduated flask. A 10 mL of 3 N HCl was further added to the crucible and heated until the solution was boiled, cooled and filtered into the graduated flask. The crucible was washed three times with deionized water and the washings were filtered into the flask. The filter paper was washed thoroughly and the washing was collected into the flask. The contents of the flasks were cooled and diluted to the mark with deionized water. A blank was prepared from the same amount of reagents except for the *Kocho* samples. The samples solutions were

transferred to polyethylene bottle. The atomic absorption spectrophotometer (AAS) was calibrated with standards. Blank, control and samples were run, respectively. The mineral contents were calculated as follows:

$$\text{mg/100g of metal content} = \frac{\square_{\square} \square \square_{\square}}{\square\square\square\square} \square\square \quad \text{.....(8)}$$

where: C_s, concentration of sample in ppm; C_b, concentration of blank in ppm; V, volume (mL) of extract and W, weight (g) of samples

3.2.4.8.11. Measurement of water-holding capacity

The water-binding capacity of the *Kocho* samples was determined according to the method reported by Adebowale *et al.* (2005). Briefly, about 2 g of *Kocho* sample (M_1) was added to 20 mL distilled water in each of 30 mL test tube (M_2) in triplicates. The samples were centrifuged at 2460 rpm for 1 h and allowed to stand for 25 min at room temperature. The supernatant water of each tube was decanted by inverting the tubes over the filter paper placed in a volumetric flask. The samples were allowed to drain for 35 min (M_3). The weight of bound water of each sample was determined by the difference between initial and final weights of *Kocho* samples and calculated using the following relation:

$$\square\square\square\square\square \; \square \; \square\square\square\square\square \; \square\square \; \frac{\square_{\square}\square\square_{\square}}{\square_{\square}} \square \; \square\square\square \text{.....} \quad (9)$$

3.2.4.8.12. Determination of phytic acid content

Phytic acid contents were determined by the method described in the literature; Latta and Eskin (1980). About 0.5 g dried *Kocho* samples were extracted with 10 mL of 0.2 M HCl for 24 h at ambient temperature and centrifuged for about 30 min at 3000 rpm in triplicates. The clear

supernatant was used for phytic acid determination. About 2 mL of Wade reagent (0.03% $FeCl_3$, and 0.3% sulfosalicylic acid) was added to 3 mL of each of the supernatant sample solution. The samples were homogenized and centrifuged for 10 min at 3000 rpm. Phytic acid (5-36 ppm) was used as a standard solution. The absorbance of each sample was measured at [illegible] 500 nm using Spectrophotometer. The content of the acid was determined using the following equation.

[illegible] ………. (10)

where A_b = absorbance of the blank, A_s = absorbance of the samples and W = weight of the samples in gram

3.2.4.8.13. Determination of tannin content

Tannin contents of the *Kocho* samples were determined by the modified vanillin-hydrochloric acid method (Price, 1978). In this study, about 0.2 g of the dried *Kocho* sample was extracted with 10 mL of 1% HCl in methanol (*v/v*) for 24 h at 30^oC in triplicates. The extract was then centrifuged at 3000 rpm for 5 min. To 1 mL extract, 5 mL vanillin reagent was added. The reaction mixture was left to stand for 20 min at 30^oC. D-Catechin (0.2–1 mg/mL) was used as a standard solution. The absorbance of the resulting solution was measured at 500 nm against the blank containing 4% HCl in methanol using spectrophotometer. Tannin concentration in mg/g was extrapolated from the standard curve. All samples were analyzed in triplicate. The values from absorbance reading were converted using equation:

[illegible] ………. (11)

where: A_s, sample absorbance; A_b, blank absorbance; d, density of solution (0.791 g/mL) and W, weight of sample in gram

3.2.4.8.14. Evaluation of sensory attributes

Sensory evaluation was conducted according to Jung *et al*. (2012), Abebe *et al.* (2006) and Ogunjobi *et al*. (2005). Sensory attributes of the *Kocho* dough and pancake like bread of fermented *Enset* without *Mandillo* (Koki) and with *Mandillo* (Kom) were evaluated by 10 panelists selected from among the local consumers. Texture, color, appearance, odor, flavor, taste, chewiness and overall acceptability were also assessed. The scores were based on a five point hedonic scale, where 5 represents excellent and 1 stands for very poor (Appendix C).

3.2.4.9. Data analysis

To study the indigenous knowledge of Shekacho society in *Enset* cultivation and *Kocho* consumption, descriptive data analysis was followed; focus group discussion data was translated and transcribed. To pronounce the names of the *Enset* plants, process and products correctly, Latin rules of writing was used. All the remaining experiments were done in triplicate and the results were expressed as Mean ± Standard Deviation (SD). The statistical analyses were done by using SPSS version 20.0 (SPSS Inc., Chicago, IL) by means of one way ANOVA followed by Duncan tests and MEGA 6 bioinformatics software was used for molecular analysis. Significance of difference was defined at $p < 0.05$ level.

Chapter 4

4. Results

4.1. Descriptive analysis of the historical background of *Enset* and its products

4.1.1. Historical background of *Enset*

Based on the responses of the study participants, there is no clear indication for how long *Enset* plant has been known to the Shekacho society and from where it was introduced. However, the entire respondents unanimously agreed that the histories of existence and usage of *Enset* may linearly coincide with the long years history of the Shekacho people. The study subjects further remarked that in the history of Shekacho society, despite some drought episodes, starvation have never been experienced (Figure 4.1).

Figure 4.1: Patchy forests and *Enset* plants in Fetafa; Sheka Zone.

4.1.2. Classification of *Enset* cultivars

One hundred fifteen *Enset* local cultivars were identified and classified as male (*Atina'o*) and female (*Maache*) types, out of which sixty one of them were physically observed from the farmers' field and virgin lands (Figure 4.2).

Figure 4.2: Cultivated (left), and wild (right) *Enset* plants.

Accordingly, one third of them classified as male types, while the remaining found to be female types, as listed down below:

Male types of *Enset* cultivars. *Aa'i-barasho, Aa'i-bosso, Aa'i-gudiro, Aa'i-noobo, Aa'i-yoobo, Aajjaro, Aajjo, Aataro, Addo, Baacci-barasho, Bado, Baraadi, Baraadi-Bosso, Barasho, Baxxato, Bosso, Bushuro, Ceella-bosso, Ceellacho, Ceella-Gemo, Gajji-bosso, Ganji-baraso, Ganji-noobo, Gayaacho, Gemo, Giillo, Goomajjo, Gudiro, Gushiro, Kabbo, Necca-Gemo, Necca-noobo, Noobo, Obaano, Ookko, Qattaano, Qefi-bosso, Shondi, Taato, Tafaro, Yaafi-bosso and Yoobo.*

Female types of *Enset* cultivers. *Aacho, Aa'i-shimmo, Aakkaro, Agane, Agani, Arakko, Ballaa-wusso, Buuti, Caaccaro, Carallo, Ceeggacho, Ciqqaro, Ganjo, Geno,Giito, Goobachi, Googashi, Gosho, Gotano, Hii-Kiwwi, Kaa'o, Kafi-qeeqqaro, Kafiyachi, Kawo, Keci, Maacaa-dami, Maaci, Maara-tiishi, Mashaa-maasho, Mashengi, Maxaaqo, Messo, Necca-barasho, Nooqo, Ogisso, Ogiyo, Oomano, Oomi, Ooqo, Qasafo, Qasi-aafo, Qebbo, Qeeqqaro, Qeraaro, Qooppiri, Qotano, Shehi, Sheki-qeeqqaro, Shicho, Shiddo, Shiimo, Shiishiri, Shiisho, Shimmo,*

Shirii, Shiwo, Shukki, Shuruddo, Shuuri, Taawo, Tisha-maati, Toobbacho, Ukkano, Waango, Wushiro, Wusso, Xeeyo, Ximbirii, Yaaho, Yeebbo, Yeqqo, Yooro and Yotto.

The interviews with key informants indicated that female types are large in number of the available cultivars, but the male types are most commonly available in the farmers' fields, huge in size, more disease and drought resistant and produce higher yields than the female ones. Some of the most abundant *Enset* cultivars and commonly used by the farmers are the following: *Baraadi-Bosso, Barasho, Carallo, Gemo, Gudiro, Maaca-dami, Maxaaqo, Noobo, Shicho*, and *Yeqqo*. The varieties of *Enset* in Sheka Zone have also been identified by color, structure and size of the different parts of the plant. Medicinal value, disease resistance and drought tolerance properties, food and fiber quality and yields are based on the cultivars of *Enset*.

Furthermore, the study participants described various parts and products of *Enset* as follows: the leaf (*maato*), leaf sheath (*yo'o*), midrib (*ooqqo*), inner soft part of pseudostem (*baqqoo*), pseudostem (*dubo*), corm/*Amicho* (*uuxo*), squeezed liquid (*quuro*), *Bulla* (*ittino*), *Kocho*, starter culture (*kiisho*), and fiber (*yi'o*). It was also learnt that the Shekacho society uses *Enset* as food sources, livestock feeds, medicine, household materials and utensils, construction materials, detergent, etc. In addition, *dubbo, fusso* and *qocho* (traditional dresses made up of *Enset* fiber for female), and *gichoo* (an ornamental made up of *Enset* fiber put on the shoulder by male) which are special heritages of Shekacho society's traditional clothes.

4.1.3. Cultivation of *Enset*

The opinions of the study subjects indicated that the male types *Enset* are primarily cultivated for food source compared to the female types. In this connection, the methods and stages of *Enset* development and cultivation followed by the Shekacho society are provided in Figure 4.3.

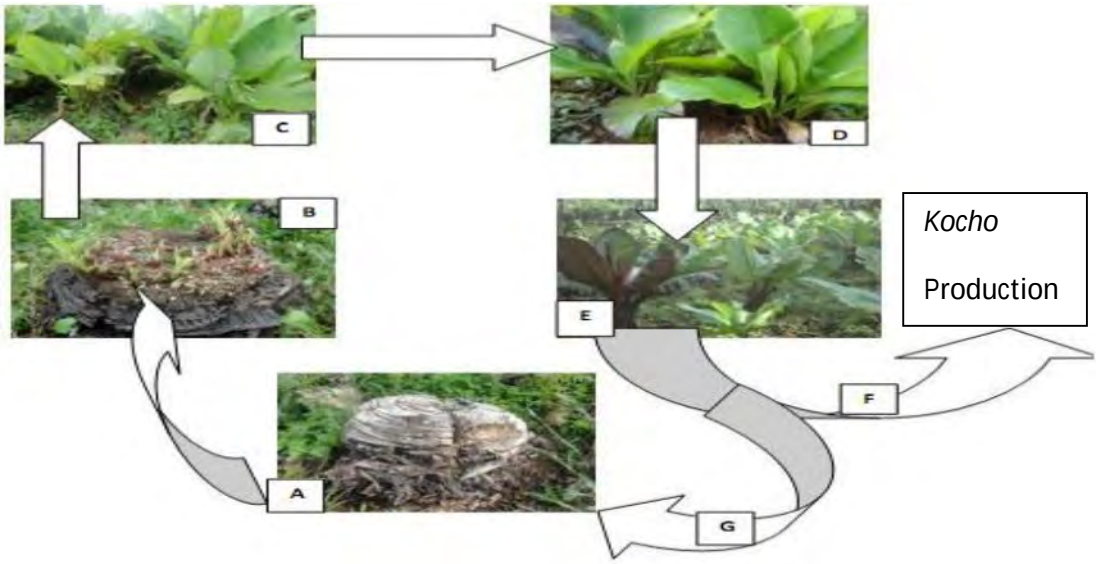


Figure 4.3: *Enset* cultivation at different development stages. A, division of the corm into four parts (*baqqo*); B, sucker formation (gaamo); C, seedlings stage (*aaco*); D, further dispersion of seedlings (*uukko*); E, final transplantation (*tokko*); G, seedling production; and F, *Kocho* production

According to the tradition of Shekacho people, the first stage in *Enset* cultivation is the removal of the pseudostem from the corm (*uuxe-maaco*), while the second stage is division of the corm into two or four parts (*baqqo*) based on its size (Figure 4.3A). The third or an optional stage is either putting the corm into the pit upside down (*duukkoo*) or it could be left intact to the ground coating with cow dung (*ibe-fuutto*). It was learnt that the number of suckers (*gaamo*) emerged per corm was found to vary from 300–400 following the traditional cultivation method in the Sheka Zone (Figure 4.3B). The suckers grow into seedlings (*aaco*) and can be transplanted after six to eight months (Figure 4.3C). The seedlings can further be dispersed (*uukko*) after a year for final transplantation (Figure 4.3D). The plant will then further transplanted (*tokko*) and allowed to grow until it matures for harvesting (Figure 4.3E), within two years of growth period. The harvesting time required by the female types varies from four to five years. However, the male types need longer periods (i.e., six or more years). In addition, the spacing required between the

two adjacent plants to grow varies from 1.5–2.0 m for male types but 1.0–1.5 m for the female types.

According to the information obtained from the experts of agricultural sectors, the favorable soil type for *Enset* cultivation is red brown. It was also confirmed that *Enset* plants grow best in the altitude ranging from 1800–3000 m above sea level. Moreover, medium to high rainfall is also preferred for healthy growth of the plants.

Regarding the sizes of *Enset* plants grown in Masha areas, the tallest plant grows to the height of about 13.1 m while the pseudostem is 2.5 m in circumference and 4 m in height. Moreover, the leaves were found to extend up to the height of 9.1 m and 1.2 m in width (Figure 4.4A). The maximum volume of the underground corm was about 1 m^3.

Figure 4.4: Parts of *Enset* plant (A) and *Mandillo* (B).

4.1.4. Share of women and *Enset* management practices

The study participants further indicated that all of the activities related to *Enset* fermentation are the responsibilities of the females. This may be mainly due to the beliefs of the society that if the

male even passes by the fermentation area, the fermentation process would fail. Furthermore, all the participants agreed that traditionally women add *Mandillo* (Figure 4.4B) when they have inadequate starter culture to enhance fermentation in order to shorten the fermentation period. Moreover, food preparations are also the responsibility of the women.

4.1.5. Products of *Enset* fermentation

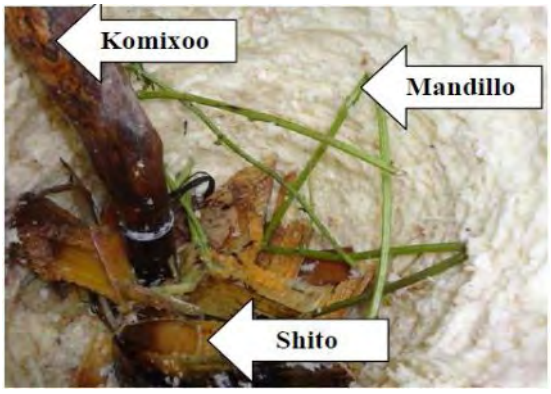
One of the observations made during the field visits was the traditional *Enset* fermentation processes. Particular attention was also given to the effect of introduction of *Mandillo* on the *Kocho* quality. Accordingly, the pulverized corm (Figure 4.5A) and the scraped pseudostem (Figure 4.5B) were performed simultaneously, mixed well and chopped with knife in the pits which resulted in unfermented mass (Figure 4.5C). Thereafter, the unfermented mass was sealed off the external air in the pits (Figure 4.5D). On the same day of *Enset* scrapping, the starter culture was also prepared from different parts of *Enset* such as *shitoo, baqqoo, ooqqo, quuro, mundiriqqoo and uuxo.* The starter culture was also sealed off the external air (Figure 4.5E and 4.5F). It was further learnt that moderate rainfall is the favorable condition for *Enset* fermentation to obtain underground water to dissolve the mixed materials of starter culture ingredients (Figure 4.5E).

A 

B 

C 

D 

E 

F 

Figure 4.5: *Enset* fermentation processes. Pulverized corm (A); Scraped pseudostem (B); Mixed and unfermented *Kocho* biomasses (C); Sealed *Kocho* biomass in a pit (D); Starter culture ingredients (E); and Sealed starter culture (F).

The semi fermented *Kocho* was then removed from the pits on the ninth day (Figure 4.6A). The biomasses were grated with the starter culture along with *Mandillo* stem and without *Mandillo* (Figure 4.6B) and mixed and put back into the pit and properly covered once again.

A  B 

Figure 4.6: A, partially fermented *Kocho*, B, Starter culture without *Mandillo* (right) and with *Mandillo* (left).

After five days, the fermented *Kocho* products were removed from the pits and squeezed while padding with *Enset* fiber. Excess water was removed from the dough and it subsequently appeared ready for consumption.

The final products showed remarkable physical quality parameters (texture, taste, ripeness, color, softness, and odor) with noticeable differences between fermentation with *Mandillo* (Figure 4.7, right) and fermentation without *Mandillo* (Figure 4.7, left). These physical quality parameters between the two products were evaluated by sensory panelists and found to be distinctly different (Table 4.17). Accordingly, the product with *Mandillo* was whiter in color, more acceptable odor and taste, ripened in a shorter time, softer and had finer texture than the product without *Mandillo*.

Figure 4.7: *Kocho* fermented without *Mandillo* (left) and with *Mandillo* (right).

4.1.6. *Enset* productivity

According to the information obtained from the respondents, the biggest matured male type *Enset* yields 200 to 250 kg *Kocho*, about 30 kg *bulla* (fresh weight) and 5 to 8 kg fiber per plant. The yield obtained from the female type is about 60 to 70 kg *Kocho*, 10 to 15 kg *bulla* and insignificant amount of fiber per plant. The productivity of *Enset* is also estimated to 62.85 to 83.33 ton ha^{-1} $year^{-1}$ on wet weight basis, while this quantity is 28.28 to 38.33 ton ha^{-1} $year^{-1}$ on dry weight basis.

The respondents had also explained that *Kocho* from the female types was softer, more palatable, better in texture and taste, matures earlier and more easily fermented than the male types. It is interesting to learn that the *Maxaaqo* variety, among the female types, is the best quality and prepared for honored guests like son-in-law, honorable personalities and leaders. *Kocho, Amicho (uuxo), eekko, Bulla (ittiino),* mixed (*bacco*) and fiber (yi'o) were also known to be the main products of *Enset* fermentation processes of the study area.

4.1.7. *Enset* based food products

The respondents indicated that from the fermented *Enset* product, pancake like bread can be made using hot plate and consumed with cabbage and animal products based sauces. The study subjects further noted that the pancake like *Kocho* can be served with other foods like *Injera* in the presence of sauce. Therefore, according to the tradition of Shekacho people, it is known that *maxino* (pancake) from *Kocho* is "the grace of the table". Other products such as *Bulla* are the best raw material in preparation of *kujjo* (soup). Furthermore, *uuxoo* (*Amicho*) can be boiled and eaten like potato. On the other hand, *uuxoo (Amicho)* from the female *Enset* type is more acceptable than the male type. *Arakko, Maxaaqo* and *Maacaa-dami* are known to be of the best quality *uuxoo* sources, while only *Ukkano* is considered from the male types of *Enset*. In addition, *kujjo* (soup), *buuxo* (porridge) *xeesso, kosso, baacuuroo, qaafoo, qoriyo, naakkasho, anjaawuro* and *buddeno* (*Injera*) are some of the cultural foods eaten along with milk, kale, cabbage, meat and different type of stews.

4.1.8. Shelf-life *Kocho*

The findings of the current study confirmed that the shelf-life of *Kocho,* produced by Shekacho women was found to extend from one month to one year by simple packaging with *Enset* leaves. However, the shelf-life can also be extended from one to two years by wrapping with *Enset* leaves and dipping into a deep water body such as slowly running river. Based on further information obtained from the respondents, *Kocho* products could also be preserved for up to six years in an air tight pit.

In addition, Shekacho people traditionally preserve *Enset* products such as *Bulla, Kocho, eekko* and *bacco* by using different spices such as *kefo (Sacred-bassil-yehabesha), toocho*

(Cymbopogon citratus), shoobbo (Lippia adoensis) and *duqquushe-waamo* (Leaf of *Allium sativum*). On the other hand, in all preservation processes, occasionally some parts or the whole products could be spoiled and in those cases the spoiled products may be identified by their color, which turns black with sour taste, unpleasant structure, mushy and pungent smell.

4.1.9. Medicinal values of *Enset*

It was traditionally believed that some of the *Enset* plant parts and their food products have medicinal values for human being and livestock. For instance, the *Kocho* are applicable to cure broken and fractured bones, joint displacement, swellings, wounds, infectious disease, diarrhea and recognized to stimulate placental discharge. According to the respondents, the most commonly used *Enset* plants for medicinal purposes include *Maacaa-dami*, *Shuuri*, and *Noobo* types.

4.1.10. Biotic factors against *Enset* productivity

Enset is attacked occasionally by different types of diseases and animal pests such as (*Kiino*) bacterial/fungal wilt (Figure 4.8), animal pests such as insects, mole rats, porcupine, wild pig, *Enset* root mealy bug and monkeys.

Figure 4.8: *Enset* plant affected by microbial wilt (locally known as *Kiino*).

The findings of the research had further indicated that the female types were more prone to the disease caused by *Kiino* than the male types. According to the respondents, *Kiino* affects the plant at all ages before inflorescence. Unlike *Kiino*, all other agents could attack both types of *Enset* before and after inflorescence.

4.2. Phytochemical evaluation, antioxidant and antimicrobial activities of *Mandillo*

4.2.1. Percent extracts recovery of *Mandillo*

The percent crude extracts recovery of *Mandillo* stem, leaves and aerial parts with 95% methanol, 70% ethanol, distilled water (DW), acetone and ethyl acetate yielded 5–12% of its dry weight (Table 4.1).

Table 4.1: Percentage yield of crude extracts of *Mandillo* stem, leaves and aerial parts

Solvents used	% of crude extracts		
	Stem	Leaf	Aerial
70% ethanol	10.5	11.5	12.0
95% methanol	9.7	11.2	11.6
Distilled water (DW)	10.8	8.7	8.1
Acetone	6.9	7.3	8.1
Ethyl acetate	6.6	5.0	7.5

The crude extract was found to be the lowest in the leaves extract with ethyl acetate (5%) and the highest in the aerial extracts with 70% ethanol and 95% methanol (12% and 11.6%, respectively), followed by the stem extract with DW (10.8%).

4.2.2. Phytochemical components of *Mandillo* leaves, aerial and stem

The results of phytochemical screening are described in Table 4.2. *Mandillo* stem showed the existence of saponins, tannins, anthraquinones, steroids, terpenoids and flavonoids, whereas, the chemical test did not reveal the presence of alkaloids.

Table 4.2: Phytochemical components determined in *Mandillo* leaves, aerial and stem extracts

Chemical groups	Test results		
	Leaves	Aerial	Stem
Alkaloids	-[a]	-	-
Saponins	+[b]	+	+
Tannins	+	+	+
Anthraquinones	+	+	+
Steroids	+	+	+
Terpenoids	+	+	+
Flavonoids	+	+	+

-[a] Not detected; +[b] qualitatively detected.

In preliminary study of this research, it was qualitatively evaluated that the addition of *Mandillo* stem decreased fermentation period of *Enset* by 5 days and improved the sensory quality parameters of *Kocho* which is in agreement with the phytochemical components and LAB growth enhancement effect of *Mandillo* stem observed (Table 4.2 and 4.10).

4.2.3. Phytochemical contents of *Mandillo*

4.2.3.1. Total phenolic contents of *Mandillo*

Aerial extracts with 70% ethanol and 95% methanol and leaves extract with 95% methanol resulted in the highest phenolic contents (101.48, 72.33 and 95.26 mg GAE/g, respectively). The maximum phenolic content in stem extraction with 70% ethanol was 35.47 mg GAE/g (Table 4.3). However, stem extract with ethyl acetate showed the lowest phenolic content of 5.69 mg

GAE/g (gallic acid equivalent/g). Most of the extracts were significantly ($p < 0.05$) different in phenolic contents, except aerial extract with acetone (68.92 mg GAE/g) and 95% methanol (72.33 mg GAE/g), as well as the aerial extract with 70% ethanol and leaves extract with 95% methanol were not significantly different (101.48 and 95.26 mg GAE/g, respectively).

Table 4.3: Total phenolic contents of *Mandillo* leaves, aerial and stem extracts

Sample type	mg GAE/g
Stem extract with ethyl acetate	$5.69^{f} \pm 1.78$
Stem extract with 70% ethanol	$35.47^{e} \pm 3.20$
Stem extract with acetone	$17.24^{d} \pm 0.09$
Leaf extract with water	$56.24^{c} \pm 0.89$
Aerial extract with Acetone	$68.92^{b} \pm 0.57$
Aerial extract with 95% methanol	$72.33^{b} \pm 5.72$
Leaf extract with 95% methanol	$95.26^{a} \pm 2.49$
Aerial extract with 70% ethanol	$101.48^{a} \pm 9.11$

Values are expressed as mean ± SD (n=3). Means for all data in the column followed by different low case letters are significantly ($p < 0.05$) different

4.2.3.2. Total flavonoid contents of *Mandillo*

Mandillo extracts resulted in substantial amount of flavonoids contents (Table 4.4). The aerial extract with 70% ethanol, the leaves and aerial extracts with 95% methanol exhibited the highest amount of flavonoid (293.25, 271. 54 and 223.52 mg QE/g (quercetin equivalent), respectively).

Flavonoid contents of the stem part were relatively low compared to the aerial and the leaves. For instance, the highest flavonoid content in the stem extract with acetone was 59.15 mg QE/g.

Table 4.4: Total flavonoids contents of *Mandillo* leaves, aerial and stem extracts

Sample types	mg QE/g
Stem extract with ethyl acetate	$2.09^{a} \pm 0.42$
Stem extract with 70% ethanol	$47.60^{b} \pm 4.42$
Stem extract with acetone	$59.15^{c} \pm 2.72$
Leaf extract with water	$69.87^{d} \pm 3.24$
Aerial extract with acetone	$69.45^{d} \pm 7.95$
Aerial extract with 95% methanol	$223.52^{e} \pm 6.28$
Leaf extract with 95% methanol	$271.54^{f} \pm 7.86$
Aerial extract with 70% ethanol	$293.25^{g} \pm 3.48$

Values are expressed as mean ± SD (n=3). Means for all data in the same column followed by different low case letters are significantly ($p < 0.05$) different

Most of the extracts of the leaves, aerial and stem of *Mandillo* showed significant ($p < 0.05$) differences, except leaves extract with water (69.87 mg QE/g) and aerial extract with acetone (69.45 mg QE/g).

4.2.4. Antioxidant activities of *Mandillo* leaves, aerial and stem

The free radical-scavenging potential of the leaves, aerial and stem extracts of *Mandillo* along with the standard ascorbic acid against 2, 2-diphenyl-1-picryl hydrazyl (DPPH) radical showed remarkable inhibition activities (Table 4.5). The extracts with 70% ethanol, 95% methanol and

distilled water of all parts of *Mandillo* showed high reduction potential of DPPH radical (IC_{50} < 50 μg/mL). Stem and aerial extracts with acetone were moderate in DPPH scavenging activities with IC_{50} (< 100 μg/mL and 200 μg/mL, respectively). However, the lowest free radical scavenging activity showed by stem extract with ethyl acetate (IC_{50} > 600 μg/mL) is not remarkable.

Table 4.5: Antioxidant activities of ascorbic acid and the leaves, aerial and stem extracts of *Mandillo* by DPPH a

% DPPH scavenging potential of ascorbic acid and *Mandillo* extracts								
Conc. mg/ mL	Ascorbic acid	95% Methanol stem	70% Ethanol stem	Ethyl acetate stem	Acetone stem	95% Methanol leaves	Water leaves	A ae
0.05	96.11^{a}±0.1.02	57.15^{e}±0.03	65.81^{b}±0.02	34.84^{h}±0.01	43.2^{g}±0.01	63.13^{c}±0.07	57.28^{d}±0.05	4
0.10	97.48^{a}±0.3.01	63.23^{e}±0.04	72.67^{d}±0.03	36.59^{i}±0.04	53.04^{g}±0.03	78.22^{b}±0.2	73.23^{c}±0.02	4
0.20	97.54^{a}±0.07	72.03^{c}±0.21	78.93^{c}±0.11	37.35^{e}±0.01	64.44^{d}±0.03	93.09^{b}±0.02	92.73^{b}±0.01	5
0.35	97.59^{a}±0.01	86.64^{c}±0.08	89.20^{c}±0.05	41.88^{f}±0.05	82.13^{d}±0.02	92.3^{b}±0.04	92.56^{b}±0.02	7
0.50	97.7^{a}±0.20	93.54^{b}±0.09	93.62^{b}±0.03	43.41^{c}±0.03	92.15^{b}±0.05	91.61^{b}±0.02	91.51^{b}±0.05	9
0.60	97.83^{a}±0.08	93.21^{b}±0.05	93.52^{b}±0.01	43.03^{c}±0.04	92.01^{b}±0.03	91.34^{b}±0.04	91.37^{b}±0.03	9

Values are expressed as mean ± SD (n=3). Means for all data in each row followed by the same low case letters
> 0.05) different

4.2.5. DNA-protective activity of *Mandillo*

The untreated bacteriophage λ DNA damaged by oxidative stress generated by the mixture of Cu (II)-ascorbic acid (Figure 4.9, lane 2). However, λ DNA treated with *Mandillo* extract (Figure 4.9, lane 3) showed clear bands as λ DNA of the control (Figure 4.9, lane 1) even though it was exposed to the oxidative stress.

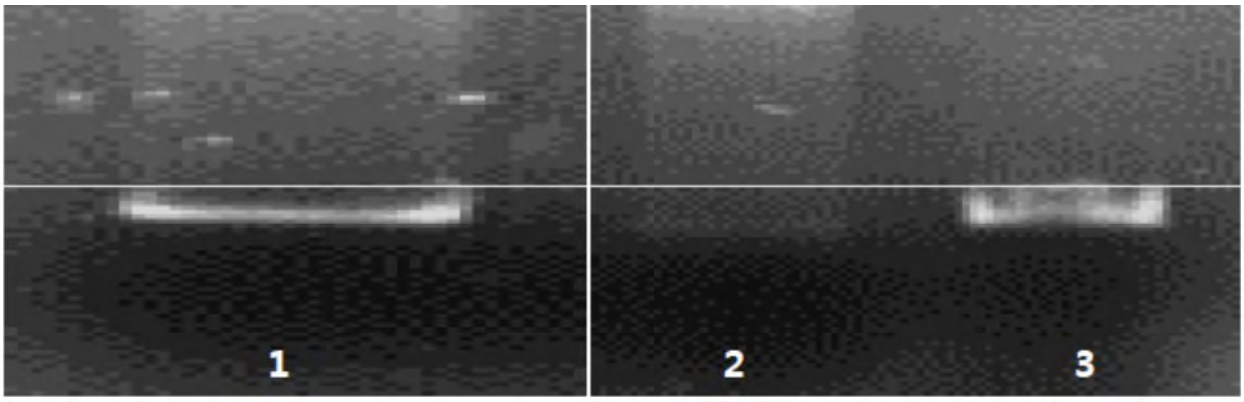


Figure 4.9: Images from agarose gel electrophoresis separation of damaged DNA and protective effect of *mandillo* extract. The numbered lane represents: Lane 1, λ DNA alone (control); lane 2, DNA plus Cu (II)-ascorbic acid; lane 3, DNA plus Cu (II)-ascorbic acid and *Mandillo* extract.

4.2.6. Antimicrobial activities of *Mandillo*

Mandillo stem extract with 70% ethanol showed promising antimicrobial activity against *E. coli* KCTC 1682, *E. faecalis* KCTC 3206, *S. aureus* KCTC 3881, *Lac. plantarum* M.B.P. 06 and *C. utilizes* KCCM 11355 at different concentrations. The stem extract totally inhibited the growth of *E. coli* KCTC 1682 and C. *utilizes* KCCM 11355 with a minimum inhibitory concentration of ≥ 15.625 µg/mL. Even though the growth of *Lac. plantarum* was inhibited at 500 µg/mL till 24 h, its growth enhanced again after 24 h (Table 4.6).

Table 4.6: Minimum inhibitory concentration and minimum bactericidal/fungicidal concentration of the stem of *Mandillo* extract with ethanol

Strains	Ethanol extract of *Mandillo* stem (μg/mL)	
	MIC	MBC/MFC
Escherichia coli KCTC 1682	≥ 15.62	≥ 15.62
Enterococcus faecalis KCTC 3206	≥ 250.00	≥ 1,000.00
Staphylococcus aureus KCTC 3881	≥ 250.00	≥ 1,000.00
Lactobacillus plantarum M.B.P. 06	≥ 500.00	≥ 1,000.00
Candida utilizes KCCM 11355	≥ 15.62	≥ 15.62

4.2.7. Fractions of extract yields and cytotoxicity of *Mandillo*

Percentage yield of fractionated *Mandillo* stem extracts are presented in Table 4.7. The yield after fractionation of the crude extracts with 70% ethanol, n-hexane, ethyl acetate, dichloromethane (DCM), butanol and distilled water showed percentage yield ranging from 4.53–52.44%. The DCM fraction was found to be with the lowest yield in the stem part (4.53%), whereas distilled water fraction of the stem part showed the highest yield (52.44%).

Table 4.7: Percentage of ethanolic extracts of *Mandillo* fractionated in different solvents

Descriptions	% of crud stem extract fractions
Mandillo crude extract with 70% ethanol (g)	10.5
Extract fractionated in distilled water (DW) (%, *w/w*)	52.44
Extract fractionated in n-hexane (n-hex) (%, *w/w*)	23.64
Extract fractionated in dichloromethane (DCM) (%, *w/w*)	4.53
Extract fractionated in ethyl acetate (EtAc) (%, *w/w*)	7.56
Extract fractionated in butanol (%, *w/w*)	11.84

Evaluation of *Mandillo* extracts showed no toxicity to raw cells at 4 µg/L in all of the fractions (Figure 4.10). Ethyl acetate fraction showed toxicity when the concentration was ≥ 10 µg/L. Likewise, all of the fractions revealed toxicity level when the concentrations were ≥ 20 µg/L. The recommended concentration (4 µg/L), is greater than that of traditionally used *Mandillo* stem by the society. The Shekacho society usually uses 100 g fresh *Mandillo* stem on average, for medium size *Enset* fermentation to produce about 100 kg *Kocho* (0.1% *w/w*). The safest concentration to apply *Mandillo* stem during *Enset* fermentation was ≤ 4 µg m/L.

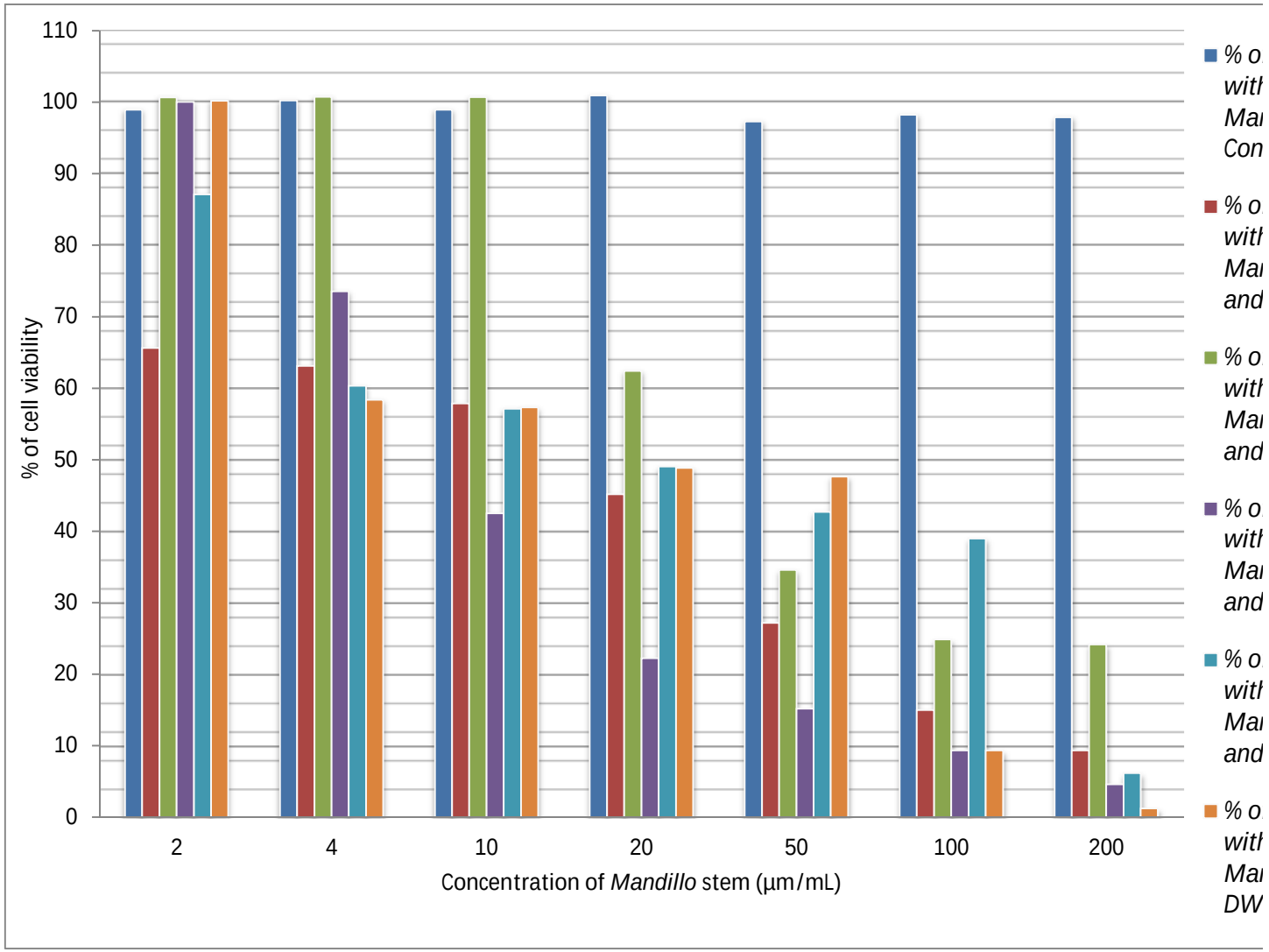


Figure 4.10: Percentage of cell viability of treated and control at different concentration of *Mandillo* stem different solvents by MTT assay

4.3. Effects of *Mandillo* during *Enset* fermentation processes in *Kocho* production

4.3.1. The pH value, titratable acidity and lactic acid and acetic acid contents

Increase in *Enset* fermentation period and the addition of *Mandillo* showed significant ($p < 0.05$) differences in pH value, titratable acidity (TA), lactic acid (LA) and acetic acid (AA) contents (Table 4.8). The effects of added *Mandillo* stem magnified the differences in fermentation profile between control *Kocho* (Koki) and experimental *Kocho* (Kom) samples (Table 4.8).

The pH values of Koki and Kom samples decreased from 4.5 ± 0.05 to 4.49 ± 0.02 and 4.75 ± 0.19 to 4.10 ± 0.02. The TA increased from 395.00 ± 5.50 to 795.00 ± 4.5 mg/100 g and 185.00 ± 0.50 to 990.00 ± 4.00 mg/100 g*;* respectively throughout the fermentation period.

The maximum difference in LA concentration between Koki and Kom was observed on the 37th day of fermentation (205.43 ± 0.01 and 418.32 ± 0.05 mg/100 g; respectively). Similarly, in the same day of fermentation, the concentrations of AA increased from 121.30 ± 0.08 mg/100 g (Koki) to 169.87 ± 0.02 mg/100 g (Kom). As a results, pH values of Koki decreased by only 0.08 and the Kom by 0.65 (Table 4.8). TA, LA and AA contents in Kom are greater by 64.34%, 103.90% and 40.04%, respectively than in Koki on the 37th day of the fermentation period.

The rates of fermentation of Koki and Kom samples were different in all the measured parameters. In the experimental groups, the fermentation process was completed after day 37. These facts may be justified, for the reason that in Koki samples, the two acids (LA and AA) productions were on progress from day 29–45 while in Kom samples the production of the acids was reduced progressively up to day 37 only. In Kom, TA and LA decreased after day 37th while AA production was on progress till day 45 (Table 4.8).

Table 4.8:The pH value, titratable acidity (TA), lactic acid (LA) and acetic acid (AA) contents in the initiatio
Kocho samples without *Mandillo* (Koki) and experimental *Kocho* samples with *Mandillo* (Kom) during *Enset* fe

Sample type	Fermentation day	pH	TA (mg/100g)	LA (mg/100g)
Initiation period (Ko)	0	$5.46^{a} \pm 0.05$	$150.00^{b} \pm 0.17$	$127.93^{b} \pm 0.2.5$
	9	$4.41^{b} \pm 0.03$	$525.00^{a} \pm 2.40$	$271.13^{a} \pm 0.13$
Control group (Koki)	11	$4.57^{eB} \pm 0.05$	$395.00^{fA} \pm 5.50$	$255.53^{bB} \pm 0.03$
	15	$4.59^{dB} \pm 0.05$	$615.00^{eA} \pm 1.50$	$195.85^{fB} \pm 0.07$
	21	$4.61^{cA} \pm 0.01$	$665.00^{dA} \pm 5.50$	$312.82^{aB} \pm 0.07$
	29	$4.67^{bA} \pm 0.01$	$680.00^{cB} \pm 6.00$	$245.52^{cB} \pm 0.06$
	37	$4.75^{aA} \pm 0.04$	$715.00^{bB} \pm 3.50$	$205.43^{eB} \pm 0.01$
	45	$4.49^{fA} \pm 0.02$	$795.00^{aB} \pm 4.50$	$215.55^{dB} \pm 0.08$
Experimental group (Kom)	11	$4.75^{aA} \pm 0.19$	$185.00^{fB} \pm 0.50$	$308.47^{eA} \pm 0.09$
	15	$4.72^{bA} \pm 0.07$	$320.00^{eB} \pm 3.00$	$264.43^{fA} \pm 0.05$
	21	$4.43^{cB} \pm 0.01$	$785.00^{dA} \pm 6.50$	$366.06^{dA} \pm 0.03$
	29	$4.25^{dB} \pm 0.04$	$1070.00^{bA} \pm 5.00$	$378.92^{cA} \pm 0.09$
	37	$4.18^{eB} \pm 0.08$	$1177.00^{aA} \pm 9.50$	$418.32^{aA} \pm 0.05$
	45	$4.10^{fB} \pm 0.02$	$990.00^{cA} \pm 4.00$	$399.26^{bA} \pm 0.05$

Values are expressed as mean ± SD (n = 3). Means for all data followed by different lower case letters within
Koki or Kom) and the same column are significantly ($p < 0.05$) different and different upper case letters in the s
column are also significantly ($p < 0.05$) different

4.3.2. Microbial counts

Aerobic mesophilic count, spore formers and coliforms significantly ($p < 0.05$) decreased in *Kocho* samples with increase in fermentation days. The differences between Koki and Kom samples in microbial dynamics were remarkably high (Table 4.9 and 2.10). Aerobic mesophilic counts of Koki and Kom were 1.26 ± 0.1 0×10^5 CFU/g and 2.5 ± 0.01×10^3 CFU/g but spore formers were below detectable level in both samples (2.30 ± 0.00×10^1 CFU/g and 4.00 ± 0.01×10^0 CFU/g; respectively). Coliforms decreased (1.6 ± 0.00×10^1 CFU/g) in Koki and Kom below the detectable level on the final fermentation period (day 45). Staphylococci were also below the detectable level throughout the fermentation period in both Koki and Kom samples.

Table 4.9: Aerobic mesophilic counts, spore formers, coliforms and staphylococci in the initiation period (Ko), c
without *Mandillo* (Koki) and experimental *Kocho* samples with *Mandillo* (Kom) during *Enset* fermentation

Sample type	Fermentation day	Aerobic plate count (CFU/mL)	Spore formers (CFU/mL)	Coliforms (CFU/mL)
Initiation period (Ko)	0	$2.80 \pm 0.15^{a} \times 10^{6}$	$1.76 \pm 0.32^{a} \times 10^{3}$	$3.65 \pm 0.07^{a} \times 10^{5}$
	9	$1.32 \pm 0.06^{b} \times 10^{6}$	$1.63 \pm 0.05^{b} \times 10^{2}$	$2.95 \pm 0.07^{b} \times 10^{5}$
Control *Kocho* Samples (Koki)	11	$2.80 \pm 0.10^{fB} \times 10^{4}$	$5.05 \pm 0.10^{b} \times 10^{2}$	$1.80 \pm 0.00^{aB} \times 10^{4}$
	15	$3.00 \pm 10.00^{bA} \times 10^{6}$	$4.90 \pm 0.10^{c} \times 10^{2}$	$1.20 \pm 0.00^{bA} \times 10^{4}$
	21	$2.75 \pm 5.00^{cA} \times 10^{6}$	$3.90 \pm 0.10^{d} \times 10^{2}$	$4.50 \pm 0.00^{cA} \times 10^{3}$
	29	$3.65 \pm 5.00^{aA} \times 10^{6}$	$7.35 \pm 0.40^{a} \times 10^{2}$	$1.20 \pm 0.00^{dA} \times 10^{3}$
	37	$5.70 \pm 1.00^{dA} \times 10^{5}$	$2.60 \pm 0.10^{e} \times 10^{2}$	$1.01 \pm 0.00^{eA} \times 10^{3}$
	45	$1.26 \pm 0.10^{eA} \times 10^{5}$	$2.30 \pm 0.00^{f} \times 10^{1\text{BDL}}$	$1.60 \pm 0.00^{fA} \times 10^{1\ \text{BDL}}$
Experimental *Kocho* Samples (Kom)	11	$3.30 \pm 0.10^{dA} \times 10^{4}$	BDL	$2.50 \pm 0.00^{aA} \times 10^{4}$
	15	$2.05 \pm 0.50^{cB} \times 10^{5}$	BDL	$7.20 \pm 0.00^{bB} \times 10^{3}$
	21	$3.55 \pm 0.50^{bB} \times 10^{5}$	BDL	$2.60 \pm 0.00^{cB} \times 10^{3}$
	29	$6.60 \pm 1.00^{aB} \times 10^{5}$	BDL	$5.90 \pm 0.00^{dB} \times 10^{2}$
	37	$2.38 \pm 0.10^{eB} \times 10^{4}$	BDL	$2.60 \pm 0.00^{eB} \times 10^{2}$
	45	$2.50 \pm 0.01^{fB} \times 10^{3}$	BDL	BDL

BDL = below detectable level. Values are expressed as mean ± SD (n = 3). Means for all data followed by diffe
within the same sample (Ko, Koki or Kom) and the same column are significantly ($p < 0.05$) different and diffe
in the same day and the same column are also significantly different ($p < 0.05$)

In the fermentation courses, the dynamics of LAB enhanced and diversified, while the growth of yeast was partially supported by the addition of *Mandillo* stem (Table 4.10). Both in Koki and Kom samples, LAB counts increased from 6.38 to 8.78 $\log_{10}$ CFU/g and from 7.54 to 8.87 $\log_{10}$ CFU/g, respectively. This showed that in the Koki and Kom samples, the population of LAB was significantly ($p < 0.05$) different throughout the fermentation period. The maximum differences between Koki and Kom were observed on the 21st day of fermentation process, which increased from 7.06 $\log_{10}$ to 8.45 $\log_{10}$ CFU/g, respectively.

Yeast counts in experimental groups increased from day 11 to 29. However, the count lowered towards the final fermentation period (day 45). Unlike the Kom samples, in Koki, yeast population increased from day 11 to 21. The population of yeast as it is described in table 4.10, the maximum difference was observed between Koki and Kom on the day 29 (5.44 $\log_{10}$ and 5,84 $\log_{10}$ CFU/g; respectively). After day 29, the ratio of yeast population of Kom to Koki decreased. This may be accounted for decrease in pH value in Kom (4.10) and increase in Koki (4.49) (Table 4.8).

Table 4.10: Lactic acid bacteria (LAB) and yeast counts in the initiation period (Ko), control *Kocho* samples wi and experimental *Kocho* samples with *Mandillo* (Kom) during *Enset* fermentation

fermentation day	LAB population (CFU/g)		Ratio of LAB (Kom:Koki)	Yeast population (CFU/g)	
0 (Ko)	$3.15 \pm 0.56^{b} \times 10^{5}$			$1.89 \pm 0.15^{a} \times 10^{4}$	
9 (Ko)	$9.47 \pm 0.21^{a} \times 10^{7}$			$1.01 \pm 0.06^{b} \times 10^{4}$	
	Kom (CFU/ml)	Koki (CFU/ml)		Kom (CFU/ml)	Koki (CFU/m
11	$3.46 \pm 0.06^{fA} \times 10^{7}$	$2.41 \pm 0.01^{fB} \times 10^{6}$	14.7:1	$1.97 \pm 0.14^{dA} \times 10^{5}$	1.15 ± 0.05^{fB}
15	$5.44 \pm 0.09^{eA} \times 10^{7}$	$8.59 \pm 0.05^{eB} \times 10^{6}$	6.6:1	$4.88 \pm 0.35^{cA} \times 10^{5}$	2.88 ± 0.50^{bE}
21	$2.83 \pm 0.13^{dA} \times 10^{8}$	$1.16 \pm 0.00^{dB} \times 10^{7}$	23.3:1	$9.77 \pm 0.30^{aA} \times 10^{5}$	7.85 ± 0.25^{aE}
29	$8.48 \pm 0.47^{bA} \times 10^{8}$	$1.16 \pm 0.77^{cB} \times 10^{8}$	7.4:1	$6.85 \pm 0.35^{bA} \times 10^{5}$	2.78 ± 0.16^{cE}
37	$9.30 \pm 0.30^{aA} \times 10^{8}$	$7.55 \pm 0.13^{aB} \times 10^{8}$	1.2:1	$2.26 \pm 0.01^{eB} \times 10^{3}$	1.59 ± 0.23^{dA}
45	$7.40 \pm 0.29^{cA} \times 10^{8}$	$6.01 \pm 0.12^{bB} \times 10^{8}$	1.2:1	$1.35 \pm 0.15^{fB} \times 10^{3}$	1.38 ± 0.75^{eA}

Values are expressed as mean ± SD (n = 3). Means for all data followed by different lower case letters within Koki or Kom) and the same column are significantly ($p < 0.05$) different and different upper case letters with th and the same row are also significantly different ($p < 0.05$)

4.3.3. Isolation and characterization of LAB and yeast

A total of 288 presumptive LAB isolates were retrieved from Ko, Koki and Kom samples (Table 4.11).

Morphological and biochemical tests showed that 288 isolates from MRS agar plates were confirmed to be LAB and all were found to be Gram positive, catalase negative, non-spore formers (red color), non-motile which showed growing in a confined stab line and acid producer from glucose. Among the 288 LAB, 212 (73.61%) were rod shaped and 76 (26.39%) isolates were cocci in shape. The growth temperature varied from 15^{o}C to 45^{o}C (Table 4.11), though the cocci isolates did not show growth at 45^{o}C. Sugar fermentation test showed, 208 (72.22%) isolates produced gas from glucose. Most of the LAB isolates exhibited facultative anaerobic nature, which may be considered as hetero and homo-fermentative based on the fermentation environment. The other 80 (28.78%) of the LAB isolates were not producing gas from glucose. The majority of these homo-fermentative LAB isolates were found in Kom samples.

The entire LAB isolates were classified into 2 genera, namely *Lactobacillus* and *Leuconostoc*. The genus *Leuconostoc* found only in the initiation period of fermentation course (Table 4.12). These results also showed positive correlation to the high concentration of lactic acid production in Kom samples (Table 4.8).

One hundred fifty five (155) isolates were also collected from from Ko, Koki and Kom samples and identified as yeast strains. Smooth, non-hairy colonies and lacking extensions at margins under stereoscopic microscope were counted and confirmed as yeast isolates.

Table 4.11: Physico-chemical characterization of LAB isolates of initiation period (Ko) control *Kocho* sam (Koki) and experimental *Kocho* samples with *Mandillo* (Kom) during *Enset* fermentation.

No of isolates	Morphology		Gas production from glucose		Growth temperature				Catalase test	Gram's reaction	KOH string test
288 (100%) LAB :	212 (73.61%)	76 (26.39%)	208 (72.22%)	80 (28.78%)	70 (24.51%)		218 (75.69%)				
					15°C	45°C	15°C	45°C	-	+	_
	Rod	Cocci	+	_	+	-	+	±			

4.3.4. Molecular identification of LAB and Yeast based on 16S and 18S rRNA gene sequences

Out of 288 LAB and 155 yeast isolates, LAB (n = 111) and yeast isolates (n = 50) were randomly selected. Before pre-screening work of LAB and yeast isolates for molecular analysis, it was observed that diversity of the isolates in Ko was less than in Koki and Kom samples. Isolates in Koki samples were less diverse than in Kom samples (Ko < Koki < Kom). Based on this observation, 14, 44 and 53 LAB (Table 4.12) and 9, 20 and 21 yeast (Table 4.13) isolates were picked from each sample for molecular analysis; respectively.

4.3.5. Phylogenetic tree and BLAST comparison of LAB and Yeast

The LAB and yeast isolates were subjected to 16S rRNA and 18S rRNA genes sequencing; respectively and phylogenetic tree analysis. The microorganisms were identified to the genus and species levels (Figure 4.11 and 4.12, and Table 4.12 and 4.13).

Clustering identical LAB sequences by sorting individual phylogenetic tree and BLAST comparison analysis grouped the 111 LAB strain into 2 genera and 10 different species. Among the 10 species, 100 strains (90.09%) showed high similarities to a genus of *Lactobacillus* and 11 strains (9.91%) to *Leuconostoc* (Table 4.12). The genus *Leuconostoc* was found only in Ko samples and the fermentation processes of *Enset* were dominated first by *Leuconostoc mesenteroides* and succeeded by *Lactobacillus* species**.**

4.3.6. Phylogenetic tree reconstruction of LAB

The LAB isolates were further sorted and identified into different species which showed similarities with *Lac. plantarum* (69.37%), *Leu. mesenteroides* (9.91%), *Lac. paracasei* (9.01%)

and *Lac. brevis* (3.60%) as dominant strains and other species (Figure 4.11). The isolates of LAB, belonging to the genus of *Leuconostoc*, were found to be hetero-fermentative that they able to produce gas from glucose and create anaerobic conditions suitable for the development and succession of *Lactobacillus* species. The present finding also revealed that some of the *Lactobacillus* species succeeded the genus *Leuconostoc* before the addition of starter culture during the final days of initiation period (day 9) (Table 4.12).

Among the identified 10 species of LAB, 2 of them were found in Ko, 6 in Koki and 8 in Kom samples. *Lac. paracasei* and *Lac. brevis* were dominantly found in Kom samples only (Table 4.12). The remaining species of LAB, however, were common to the Koki and Kom samples. However, *Lab plantarum* predominated both *Kocho* samples (Koki and Kom). In view of the existence of high population and diversified species of LAB in Kom, the 2 species are exhibiting special characteristics. For instance, *Lac. paracasei* is the highest in population (90%) during final day of fermentation (day 45) when the pH of Kom was 4.10.

Table 4.12: Percentage (%) of lactic acid bacteria found in the initiation period (Ko), control *Kocho* samples wi and experimental *Kocho* samples with *Mandillo* (Kom) during *Enset* fermentation

Type of bacteria[a)]	Total strain (111)		Ko (14)		Koki (44)	
	No. of strain	% of strain	No. of strain	% of strain	No. of strain	% of strain
Lactobacillus brevis	4[a]	3.60	-[b]	-	-	-
Lac. paracasei	10	9.01	-	-	-	-
Lac. plantarum	77	69.37	3	2.70	41	36.94
Leu. mesenteroides	11	9.91	11	9.91	-	-
Unidentified LAB strains	9	8.12	-	-	3	2.70

[a)], Type of bacteria: indicate bacteria exhibiting the highest identity with a microorganism isolated in this study
[b)], no strains found

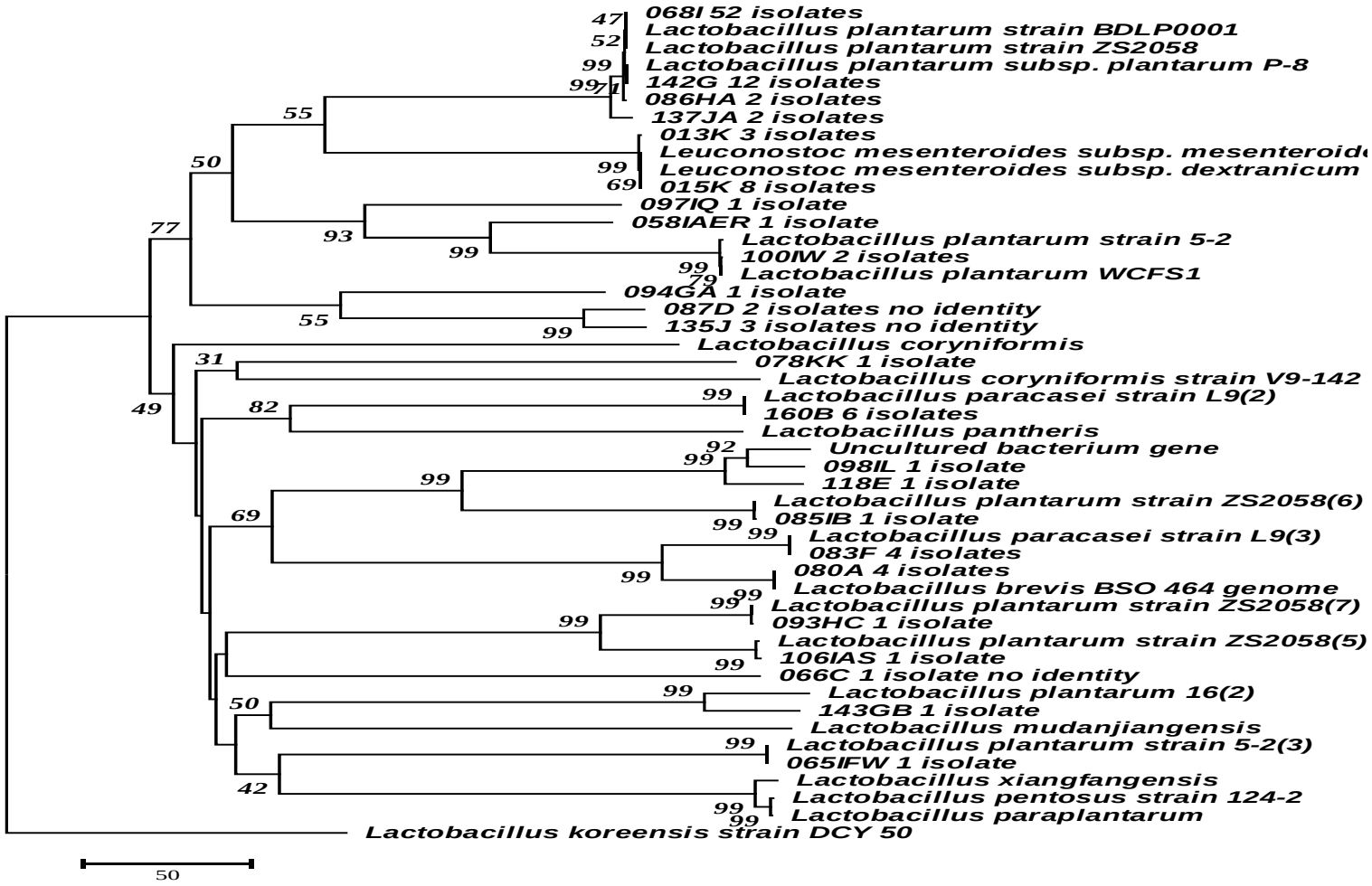


Figure 4.11: Phylogenetic tree of LAB isolated from fermented *Kocho*. The evolutionary history was inferred using method. The confidence probability estimated using the bootstrap test (1000 replicates). There were a total of 133 dataset. The phylogenetic tree was reconstructed using MEGA 6. The sequence of *Lac. koreensis* was used as an out-gr

4.3.7. Phylogenic tree reconstruction of yeast

The 18S rRNA gene sequences analysis of 50 isolates of yeast, phylogenetic tree analysis and BLAST comparison showed that the isolates were more or less evenly distributed in both Koki and Kom samples (Figure 4.12 and Table 4.13). The majority of the yeast strains were among the *Candida boidinii* (30%), *Wickerhamomyces* sp. (16%), *Pichia fermentans* (16%), *Williopsis* sp. (10%) and unidentified yeast (Picha and Fungi) strains (26%). However, *Pichia fermentans* was more dominant in Kom than in Koki samples whereas the unidentified fungal group was observed only in Ko and Koki. The unidentified fungi are expected to be new strains where further identification work is required.

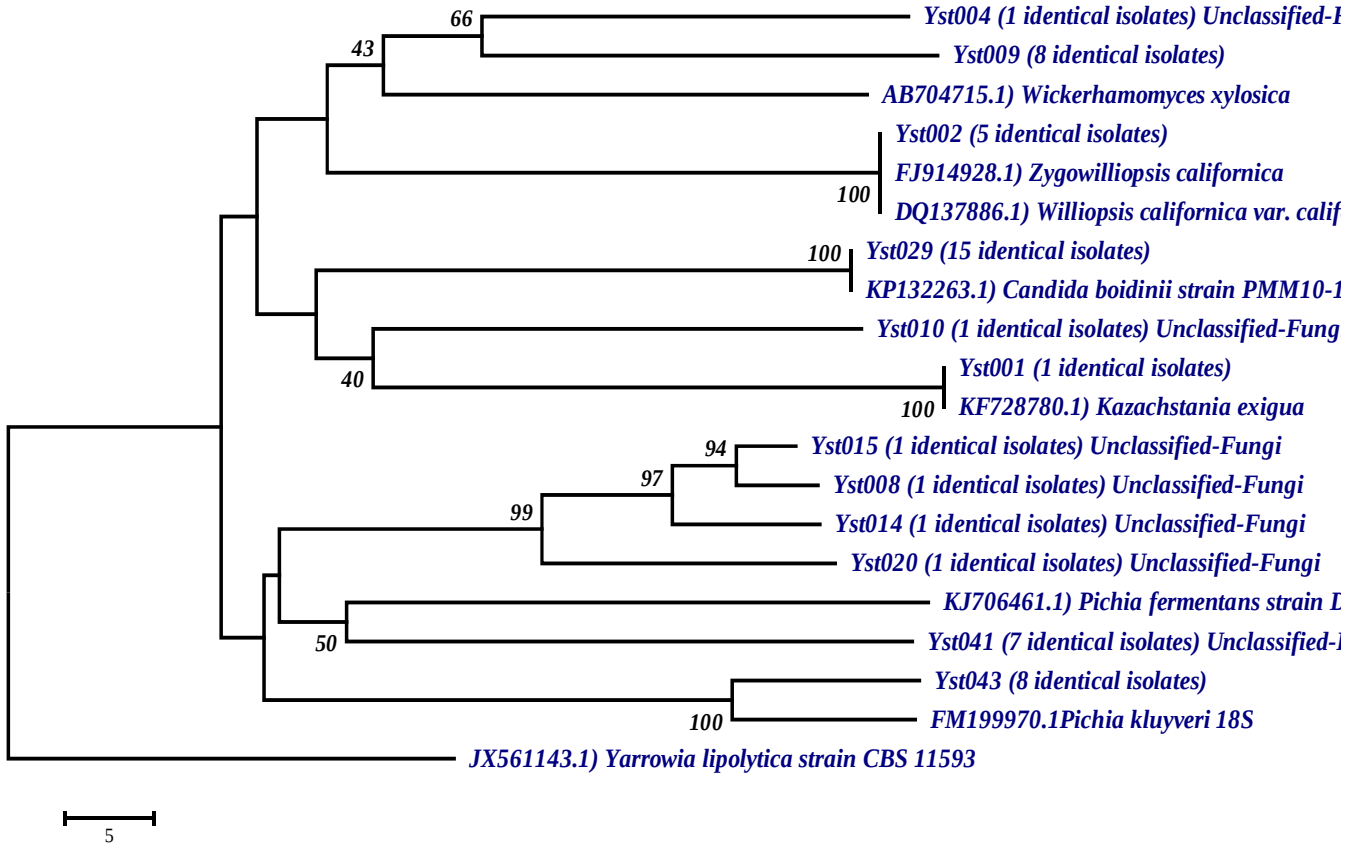


Figure 4.12: Phylogenetic tree of yeast isolated from fermented *Kocho*. The evolutionary history was inferrec Joining method. The confidence probability estimated using the bootstrap test (1000 replicates). There were a tc the final dataset. The phylogenetic tree was reconstructed using MEGA 6. The sequence of *Yarrowia lipolytic* group representative

Table 4.13: Percentage (%) of yeast distributed in the initiation period (Ko), control *Kocho* samples without experimental *Kocho* samples with *Mandillo* (Kom) during *Enset* fermentation

Name of the groups	No. isolates	% of isolates	Number of isolates /sample groups/fermentation day										
			Ko (9 isolates)	Koki (20 isolates)							Kom (21 is		
			0-9	11	15	21	29	37	45	%	11	15	2
Pichia Fermentans	8	16	-	-	-	-	1	-	-	2	-	-	3
Pichia	7	14	-	-	-		2	1	1	8	-	-	1
Wickerhamomyces Sp. ES22S03	8	16	1 [a]	1	-	2	-	-	-	6	2	2	-
Candida Boidinii	15	30	-	-	-	-		4	4	16	-	-	-
Williopsis_Sp.	5	10	5	-	-	-	-	-	-	-	-	-	-
Kazachstania Exigua	1	2	1	-	-	-	-	-	-	-	-	-	-
Fungi	6	12	2	1	2	1	-	-	-	8	-	-	-

[a)], Type of yeast indicate yeast exhibiting the highest identity with a microorganism isolated in this study.
[-)], no strains found

4.3.8. Food (*Kocho)* composition

The results of proximate analysis of the control (Koki) and experimental (Kom) samples are presented in table 4.14. Moisture content of wet samples showed high values of 79.92 to 82.12% throughout fermentation period of day 0 to 90 (data were not shown). The fermentation bags and jars helped to retain the moisture content that may otherwise have been removed through leakages and evaporation mechanisms. The retention of moisture might play great role in solving the problem of mineral and other water soluble nutrient losses during *Enset* fermentation processes.

The ash contents of Kom on day 37 showed significant ($p < 0.05$) difference from day 75 and 90, however the difference was not significant ($p > 0.05$) between day 75 and 90. In Kom the content of ash between day 37 and 90, lowered from 2.11 to 1.63%. The ash content in Koki showed significant ($p < 0.05$) change, even though the changes were irregular (Table 4.14).

Protein contents showed significant differences ($p < 0.05$) with the addition of *Mandillo* and increase in fermentation period. Accordingly, protein contents of Koki and Kom samples increased from 3.68% (Koki) to 3.95% (Kom) on the day 90, respectively, as a result of the addition of *Mandillo*. Koki and Kom samples also showed improvements in protein contents from 3.24 to 3.68% and 3.59 to 3.95, respectively, as a result of increase in fermentation period.

Fiber contents showed significant ($p < 0.05$) differences with the addition of *Mandillo* and because of fermentation period differences. The fiber contents of Koki and Kom lowered from 5.56% in Koki and to 3.96% in Kom due to *Mandillo* addition. On the other hand, the increase in fermentation day lowered the fiber content from 7.92 to 6.25% in Koki and 5.56 to 3.96% in

Kom, respectively. Both Koki and Kom showed the highest amount of carbohydrate contents of 79.06 and 81.79% on dry mass bases; respectively (Table 4.14).

In the present study considerable amount of total fat found in both Koki and Kom. The content in Koki significantly increased from 0.73% (day 37) to 0.85% (day 90). Maximum fat content in Kom was also found as 0.84% (day 75).

Table 4. 14: Proximate composition of control *Kocho* samples without *Mandillo (Koki) and experimental Mandillo* (Kom)

Fermentation day/sample type	% Moisture (dry mass)	% Ash	% Protein	% Fiber	% Fat
37 kom	$9.14^{aA} \pm 0.00$	$2.11^{aA} \pm 0.08$	$3.59^{cA} \pm 0.12$	$6.25^{aB} \pm 0.12$	$0.73^{bA} \pm 0.01$
75 Kom	$8.80^{bB} \pm 0.01$	$1.75^{bB} \pm 0.10$	$3.88^{bA} \pm 0.31$	$4.72^{bB} \pm 0.32$	$0.84^{aA} \pm 0.00$
90 Kom	$7.95^{cB} \pm 0.01$	$1.63^{bB} \pm 0.07$	$3.95^{aA} \pm 0.09$	$3.96^{cB} \pm 0.21$	$0.72^{bB} \pm 0.00$
37 koKi	$8.34^{cB} \pm 0.01$	$2.00^{bB} \pm 0.07$	$3.24^{cB} \pm 0.02$	$7.92^{aA} \pm 0.09$	$0.73^{bA} \pm 0.02$
75 KoKi	$9.30^{aA} \pm 0.02$	$1.95^{cA} \pm 0.03$	$3.52^{bB} \pm 0.07$	$6.67^{bA} \pm 0.24$	$0.75^{bB} \pm 0.01$
90 KoKi	$8.80^{bA} \pm 0.01$	$2.05^{aA} \pm 0.07$	$3.68^{aB} \pm 0.07$	$5.56^{cA} \pm 0.07$	$0.85^{aA} \pm 0.03$

Values are expressed as mean ± SD (n = 3). Means for all data in the same sample types, followed by different lo same column are significantly ($p < 0.05$) different. The same upper case letters in the same day and in the same and Koki is not significantly ($p > 0.05$) different

4.3.9. Mineral contents

Koki and Kom samples contained reasonable quantities of minerals (Table 4.15) such as Ca (9.86 ± 0.99 mg/100 g and 10.11 ± 0.45 mg/100 g), Fe (2.11 ± 0.30 mg/100 g and 2.16 ± 0.38 mg/100 g), Zn (1.99 ± 0.18 mg/100 g and 1.73 ± 0.13 mg/100 g) and Cu (0.13 ± 0.03 mg/100 g and 0.16 ± 0.04 mg/100 g; respectively

Table 4.15*:* Mineral contents of control *Kocho* samples without *Mandillo* (Koki) and experimental *Kocho* samples with *Mandillo* (Kom)

	Mineral contents			
Kocho samples	Fe	Ca	Zn	Cu
Koki (mg/100g)	2.11 ± 0.30	9.86 ± 0.99	1.99 ± 0.18	0.13 ± 0.03
Kom (mg/100g)	2.16 ± 0.38	10.11 ± 0.45	1.73 ± 0.13	0.16 ± 0.04

4.3.10. Water holding capacity

Maximum water holding capacity of Koki and Kom on dry weight bases showed 8.55% and 6.37%, respectively (Table 4.16).

4.3.11. Anti-nutrient contents

Anti-nutrient contents of Koki and Kom samples significantly ($p < 0.05$) changed in both cases with changes in fermentation day (Table 4.16). Phytic acid lowered from 5.83 to 4.11 mg/g in Koki and from 4.95 to 4.33 mg/g in Kom samples. Even though, the tannins contents of Koki and Kom lowered from 1.12 to 1.08% and from 1.09 to 1.06%; respectively. The difference due to the addition of *Mandillo* was not significant ($p > 0.05$).

Table 4.16: Water holding capacity, phytic acid and tannin contents in control *Kocho* samples without experimental *Kocho* samples with *Mandillo* (Kom)

Sample	Koki			Kom	
	Day 37	Day 75	Day 90	Day 37	Day 75
Water holding capacity (%)	$6.69^{bA} \pm 0.09$	$8.55^{aA} \pm 0.09$	$6.48^{cA} \pm 0.12$	$6.22^{bB} \pm 0.10$	6.37^{aB}
Phytic acid (mg/g)	$5.83^{aA} \pm 0.02$	$4.27^{bB} \pm 0.01$	$4.11^{cB} \pm 0.00$	$4.95^{aB} \pm 0.01$	4.43^{bA}
Tannin (%)	$1.12^{aA} \pm 0.01$	$1.10^{abA} \pm 0.02$	$1.08^{bC} \pm 0.02$	$1.09^{B} \pm 0.02$	1.08^{aA}

Values are expressed as mean ± SD (n = 3). Means for all data in each Koki and Kom independently, followed b letters in row are significantly ($p < 0.05$) different and the same upper case letters on the same day and in significantly ($p > 0.05$) different

4.3.12. Sensory attributes of *Kocho*

Sensory evaluation of overall acceptability test rated using 5 point hedonic scale (Abebe *et al*., 2006) showed remarkable differences between Koki (3.63) and Kom (4.92) (Table 4.17). Most of the measured sensory attributes showed significant ($P < 0.05$) differences between Kom and Koki except for appearance and taste of *Kocho*, taste and chewiness of pancake like bread. According to the panelists, *Kocho* pancake obtained from fermentation of *Enset* in the presence of *Mandillo* was fine in texture, whiter in color, has attractive appearance, good odor and flavor. Even though the taste and chewiness of the bread were not significantly ($P > 0.05$) different, the taste and chewiness of the pancake like bread made from Kom (rated as 5.00 and 4.33) compared to Koki (rated as 4.33 and 3.67; respectively).

Table 4.17: Sensory attributes results of control *Kocho* samples fermented without *Mandillo* (Koki) and experi
fermented with *Mandillo* (Kom) and the starter culture

Kocho			Pancake	
Measured parameters	Samples	Mean ± SD	Measured parameters	Samples
	Koki	3.33^{b} ± 0.58		Koki
Tecture	Kom	5.00^{a} ± 0.00	Texture	Kom
	Koki	3.33^{b} ± 0.58		Koki
Color	Kom	5.00^{a} ± 0.00	Color	Kom
	Koki	4.00^{a} ± 0.00		Koki
Appearance	Kom	4.33^{a} ± 0.58	Appearance	Kom
	Koki	3.33^{b} ± 0.58		Koki
Odor	Kom	5.00^{a} ± 0.00	Odor	Kom
	Koki	3.67^{b} ± 0.58		Koki
Flavor	Kom	5.00^{a} ± 0.00	Flavor	Kom
	Koki	3.67^{b} ± 0.58		Koki
Taste	Kom	4.00^{b} ± 0.00	Taste	Kom
Overall acceptability	Koki	3.33^{b} ± 0.58		Koki
of *Kocho*	Kom	5.00^{a} ± 0.00	Chewiness	Kom
				Koki
Overall acceptability of Pancake				Kom

5 excellent, 4 Very good, 3 Good, 2 poor and 1 bad. Values are expressed as mean ± SD (n = 3). Means for all da
different lower case letters in column for each parameter are significantly ($p < 0.05$) different

Chapter 5

5. Discussion

In the study area, drought and starvation have never been experienced probably because of the established tradition of *Enset* plant cultivation as a major source of food, as well as to the presence of dense forests and fertile lands (Figure 4.1). The Shekacho society group *Enset* cultivars into male and female. The classification of *Enset*, as male and female were based on the structure, size, food quality and tolerance to stresses; but has nothing to do with biological reproductive systems of the plants. Similar to other zones that cultivate *Enset* plants (Tsehaye *et.al*, 2006), the varieties in Sheka Zone have also been identified by color, structure and size of the different parts of the plant. Medicinal value, disease resistance and drought tolerance properties, food and fiber quality and yields are based on the cultivars of *Enset*.

Concerning *Enset* cultivation, unlike the report by Brandt *et al.* (1998) who indicated an altitude requirement starting from 1100 m above the sea level for favorable growth of *Enset* plants; in Sheka Zone, its growth altitude is ranging from 1800 to 3000 m above the sea level where medium to high rainfall (1201–1800 mm) is available for healthy growth of the plants. In *Enset* cultivation, a large number of seedlings could emerge from the underground corm. Contrary to the report by Kanshie (2002), who reported about 200 suckers to emerge per *Enset* corm, it was learnt that the number of suckers (*gaamo*) emerged per corm was found to vary from 300–400, following the traditional cultivation method in the Sheka Zone (Figure 4.3B). The differences in number of suckers may be as a result of differences in *Enset* cultivars, rainfall, altitude and soil fertility.

Enset ferments to produce starchy foods named *Kocho* and *Bulla*, the activities related to *Enset* fermentation high labor demanding and time taking activities. In the research area, however, every activity related to *Enset* fermentation are the responsibilities of the females. Even though, the beliefs of the society are that the fermentation process would fail if the male even passes by the fermentation area, these activities shouldn't be entirely left for females. As the productivity of *Enset* is much better than any other crop in Ethiopia and very important for food security, the harvesting activities must be supported by the males and technology, where the attention of the government bodies is very important. *Kocho* productivity of very large *Enset* plant, in the study area was estimated to 28.28 to 38.33 ton ha^{-1} $year^{-1}$ on dry weight basis. This can be concluded that the yields from the current study area were by far better than that reported by Tsegaye and Struik (2001) compared to any other crops in the country.

One of the drawbacks in *Enset* fermentation is the occasional spoilage of *Kocho* and in those cases the spoiled products may be identified by color, which turns black with sour taste, unpleasant structure, mushy and pungent smell (Gashe, 1987a). According to the Shekacho society, the probles may be solved by different means of food preservation, like adding spices, sealing in an air tight pit and keeping in air tight materials, which is in agreement with the previous report by Holzapfel (2002). On the other hand in the study area, *Enset* plant may be affected by different types of diseases and animal pests like bacterial/fungal wilt locally named *Kiino*. According to Addis *et al.,* (2008), *Enset* root mealy bug or bacterial wilt is a country-wide problem of *Enset* cultivation which needs serious attention of researchers and the government.

In general, the high yielding potential and drought tolerance nature of *Enset* in the study area was vital for the conservation of forest and proper management of land. *Enset* cultivation has been specified as one of the most important crops in the food security strategic plan of the country. *Enset*, a multipurpose plant, is used for production of food, livestock feeds, medicine, making of household materials, utensils, construction materials and detergents.

Production of *Kocho* is time taking processes unless optimum fermentation environment is created. The addition of *Mandillo* by the Shekacho women was observed when they have inadequate starter culture to enhance fermentation processes and reduce the fermentation period. These are the main finding and best practices to be adopted by the remaining parts of the societies of the country and the world.

Different studies showed that plant materials are added to food as spices and preservatives to improve flavor, odor, taste, color and to increase safety, quality, and shelf-life of the food (Sahlin, 1999; Stuart, 1986). The components of such plants may possess characteristic flavors, antioxidant, anticancer, antitumor and antimicrobial activities (Patel, 2011; Smid *et al*., 1999). Phenolic compounds in general have strong positive affection with antioxidant activities and may prevent food degradation (Proestos *et al*., 2013; Alsabri, 2012; Vladimir-Knežević *et al*., 2011). According to Wu *et al*. (2009), aromatic plants are rich in phenolic compounds. They are well known as natural food preservatives and have positive health impacts due to their antioxidant and antimicrobial properties (Shan *et al*., 2007).

For instance, saponins bind to bile salt and cholesterol in the intestinal tract and cause a reduction of blood cholesterol by preventing its re-absorption (Aja *et al*., 2015). The study made

by Wadood *et al*. (2013) showed terpenoids exhibiting various important pharmacological effects like antibacterial activity. Phenols, flavonoids and tannins are known for their antimicrobial and antioxidant activities (Watal *et al*., 2014; Ncube *et al.* 2008). In the evaluation of antimicrobial activities of *Mandillo* stem, the inhibitory effects on the growth of some Gram negative bacteria and yeast was confirmed at very low concentration. These inhibitory effects of *Mandillo*, in turn are indication of the existence of the bioactive compounds described in Table 4.2 in the plant parts. On the other hand, the plant material enhanced the growth and diversification of lactic acid bacteria (LAB). Moreover, in the preliminary study of this research, it was qualitatively evaluated that the addition of *Mandillo* stem shortened the fermentation of *Enset* by about 5 days and improved the sensory quality parameters of *Kocho* which is in agreement with the LAB growth enhancement effect of *Mandillo* stem.

The flavonoids contents of *Mandillo in* the present study are found in the same range of pigeon-pea leaves extracts with ethanol and water (293.45 and 146.32 mg QE/g; respectively) (Wu *et al*., 2009). The flavonoid contents of *Mandillo* may improve the quality of food when applied as a starter culture adjunct, for flavonoids also have been well established as potent antioxidant and antimicrobial compounds (Fang *et al*., 2007).

The antioxidant activity of *Mandillo* was studied by DPPH assay and showed strong DPPH radical scavenging power. According to Oloyede and Ogunlade (2013), extracts with the lowest IC_{50} values have the highest DPPH radical scavenging activities. *Mandillo* exhibited similar scavenging potential of DPPH radical when compared to the previous study on the plant of the same genus, *Crassocephalum bauchiense* (IC_{50} = 28.57–389.38 μg/mL) (Mouokeu, 2014). Moreover, the extracts showed stronger antioxidant potential than pigeon-pea leaves extracted with ethanol and water (IC_{50} = 242.01 and 404.91 μg/mL; respectively) (Wu et al., 2009). The

maximum amount of total phenolic and total flavonoids contents (101.48 mg GAE/g and 293.25 mg QE/g, respectively), might indicate the contribution of *Mandillo* to the antioxidant activities observed in Table 4.5. According to Vladimir-Knežević *et al.* (2011) and Stanojević (2009), antioxidants can delay, inhibit or prevent the oxidation of oxidizable materials by scavenging free radicals when added to food stuffs.

Green plants and herbs have been well studied for their antioxidant activities by a number of investigators (Proestos *et al*., 2013; Alsabri, 2012; Wu *et al*., 2009). According to Aja *et al*. (2015), polyphenols are strong antioxidants that prevent oxidative damage to biomolecules such as DNA. They are also linked to slowing cancer, aging, atherosclerosis and inflammation (Dai and Mumper, 2010). The study made by Sultana (2012) showed that dietary supplementation of flavonoids reduce the oxidative damage to cell membrane lipids, proteins and nucleic acids due to strong quenching property of free radicals (Doughari, 2012). This property of plant materials is inline with result of the present study with DNA protecting effect of *Mandillo*. The study conducted by Yin *et al*. (2013) also showed that antioxidant capacity of plants has been linked to many health promoting properties by scavenging free radicals which are involved in different disorders such as cancer.

The study of the microbial dynamics of lactic acid bacteria (LAB) and other micro-organisms showed that the growth of LAB suppressed during the first days of the addition of *Mandillo* (day 11) which may be as a result of environmental adaptation to the added *Mandillo* and enhanced toward the final days of fermentation period while the growth of other microorganisms linearly decreased. The results described in tables 4.9 and 4.10 are in agreement with the relatively low populations of the microorganisms and LAB count of 11^{th} day fermentation period of *Enset,* respectively. These findings, therefore, confirmed that the addition of *Mandillo* during *Enset*

fermentation creates a favorable environment for the growth of LAB by inhibiting the growth of some foodborne pathogens and spoiler microorganisms. Such fermentation environment may minimize competition for space and nutrient to support the growth of LAB and some yeast species. This in turn speeds up the rate of fermentation to improve organoleptic quality of *Kocho* produced and increases its shelf-life as noted by Onda (2003).

Cytotoxicity study of *Mandillo* showed the recommended concentration of 4 µg/mL, is greater than that of traditionally used *Mandillo* stem by the society. The Shekacho society usually uses 100 g fresh *Mandillo* stem on average, for medium size *Enset* fermentation to produce about 100 kg *Kocho* (0.1% *w/w*). Therefore, the safest concentration to apply *Mandillo* stem during *Enset* fermentation is $\leq$ 4 µg/mL.

In natural fermentation, there are different factors affecting microbial dynamics, diversities, successions and activities. Such factors are geographical location, seasons of fermentation, types of food items or raw materials, biochemical and biophysical nature of the existing environment (Zhou, 2012).

For instance, in South Korea and Philippines, Sikhae and Burong, respectively made by mixing salted fish and cereals of which fermentation is initiated by *Leu. mesenteroides* (Rhee *et al*., 2011). LAB strains in Korean Kimchi, *Leu. mesenteroides*, *Lac. plantarum* and *Lac. kimchii* were found to be dominant (Wadamori, 2014; Yoon, 2000). In Ethiopia, Hunduma and Ashenafi (2011) and Gashe (1987a) isolated different species of lactic acid bacteria from *Kocho* dough and reported that the isolates were Gram positive, catalase negative, non-spore forming and non-motile. As it was reported by these researchers, microbial diversity within *Kocho* dough may not be identical in different environments. The results in the present study showed differences

between Koki and Kom samples in microbial populations, succession and diversities as a result of changing the environment by adding *Mandillo* to the experimental groups (Kom). The diversified beneficial LAB species in Kom may have synergetic effects to inhibit foodborne pathogenic and spoiler microorganisms. Numerous substrates with medicinal and nutritional values which make fermented food wholesome were also reported (Perdigon *et al*., 1995). When food safety is a major concern, antimicrobial substances like bacteriocins and others produced by some LAB may play a great role.

From the differences observed between Koki and Kom samples in pH value and acids productions, we may conclude that the addition of *Mandillo* enhances the rate of fermentation of *Enset*. As it has been described by Anthon (2011), good fermentation practices may maximize safety, palatability, shelf-life and overall sensory attributes of food products. Food is also said to be microbially safe when the pH is less than 4.4, where most of foodborne spoilers and pathogenic bacteria could not survive or totally inhibited.

The present study results are in agreement with the previous findings reported by Idris (2001) and Gashe (1987a). However, the inhibition of staphylococci may not be related to the effects of *Mandillo* added; rather it may be attributed to the fermentation environments that could create unfavorable conditions to the growth of the microorganisms as detailed in the findings of Abegaz (2007), Ashenafi (2006) and Gashe (1987a). According to Onda (2003), pathogenic and spoiler microorganisms may be inhibited by fermentation bioactive products and reduction of pH during the course.

One of the reasons for the decrease in microbial counts was a result of the increase in the fermentation days. The addition of *Mandillo* may also be remarked as a synergic effect and

hurdle for the inhibition of the growth of foodborne pathogens and spoiler microorganisms. It was also observed that *Mandillo* inhibited the growth of coliforms below the detectable level towards the final days of fermentation period. Similar to what was reported by Idris (2001), in the present finding, the spore former count decreased to $4.00 \pm 0.01 \times 10^0$ CFU/g in Kom samples, which is not significant in number. This result also in agreement with the antimicrobial activities of *Mandillo* stem undertaken as part of this study results that the stem extracts showed a minimum inhibitory concentration and minimum bactericidal and fungicidal concentration of ≥ 15.63 µg/mL against *E. coli* KCTC 1682 and *Candida utilizes* KCCM 11355, respectively.

The differences between Koki and Kom treatments in pH values and organic acids concentrations (TA, LA and AA) are also the main factors for the quality differences between the two *Kocho* products. These differences in pH value and acidity are also responsible for food safety, tart (sharp-tasting) and shelf-life (Anthon, 2011). Therefore, the present study results reveale that the additions of *Mandillo* and increase in fermentation period are the two important factors for the improvement of fermented *Kocho* qualities.

In the present study, in general, the population of yeast was not as high as the previous findings by Karssa *et al.* (2013), Hunduma and Ashenafi (2011), Gashe (1987a) and Hesseltine (1985). This could be due to the fact that the current fermentation processes took place in air tight environments than the previous study conducted by Gashe (1987a) and others. According to Gashe (1987a), the fermentation processes took place in less anaerobic pits instead of the current modified environment maintained in the jars sealed with polyethylene bags. This condition may also negatively influences the productions of alcohol from sugar which further changed to acetic acid by the activities of *Acetobacter* Sp in aerobic environment as explained by Karssa *et al.* (2013), Hunduma and Ashenafi (2011), Yamada (1997) and Gashe (1987a**)**. On top of these, the

decrease in aerobic mesophilic count, spore formers, coliforms and inexistence of staphylococci, during fermentation courses, minimizes the competitions for nutrients among LAB and the remaining microorganisms and created favorable environment for vigorous multiplication of LAB as also studied by Hibbing *et al.* (2010**)**.

Isolation and identification of LAB and yeast showed the dynamic, diversity and succession profiles of the microorganisms. All the isolates were identified on the basis of their morphological, cultural, physiological, biochemical parameters.

Hunduma and Ashenafi (2011) and Gashe (1987a) isolated lactic acid bacteria from *Kocho* dough in different parts of the country and reported that all the isolates were Gram positive, catalase negative, non-spore forming and non-motile. Likewise, in our present study, we identified 288 isolates of LAB from 17 *Kocho* samples of initiation period (Ko), control (Koki) and experimental (Kom) groups. All the strains were Gram positive, non-spore forming and all of them were showing catalase negative. Ashenafi (2011) reported that lactic acid bacteria are facultative anaerobes with a preference of anaerobic conditions. Aerobic organisms that have the enzyme catalase breakdown hydrogen peroxide as in following reaction.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

In our investigation, majority (288) of the isolates didn't form bubble in 3% of H_2O_2 solution that showed the absence of catalase enzyme and these were among the genuera of lactic acid bacteria. Yirmaga (2013) isolated different strains of lactic acid bacteria from *Kocho*. They reported that all the strains were non-motile. Similarly, in the present study, all of the 288 strains that we isolated from different *Kocho* samples were non-motile (growing in a confined stab line). Filya (2007) reported that during fermentation of glucose it was reported that acid was produced

without gas in the Durham's tube. In the present investigation, the production of acid by 288 isolates during fermentation of glucose was evidenced by the change in color of the sugar media from red to yellow. Eighty (80) of the isolates were without production of any gas in the Durham's tube, where 208 isolates produced gas. These bacterial cultures were found to be homofermentative (80 isolates) and hetero-fermentative (208 isolates), respectively. In the present study, including the above identification techniques, 16S rRNA (LAB) and 18S rRNA (yeast) gene sequences analysis and phylogenetic tree reconstruction were applied for further information of LAB and yeast to the species level.

Among the 111 identified LAB species, *Lac. plantarum* (69.37%), *Leu. mesenteroides* (9.91%), *Lac. paracasei* (9.01%) and *Lac. brevis* (3.60%) were the dominant strains in the *Kocho* samples. *Lac. paracasei* and *Lac*. *brevis* were dominantly found in Kom samples. The existence of high population of facultative anaerobic LAB, such as *Lac. plantarum* (63.96%), may contribute to the high concentrations of LA production in the anaerobic condition in both Kom and Koki. This result can be justified as it was reported by Filya (2007) that homofermentative LAB like *Lac. plantarum* and others enhance rapid production of lactic acid and the consequent decrease in pH. As it was also observed from 16S rRNA gene sequences evaluation, the species found dominantly in Kom, that exhibited the highest identity with *Lac. paracasei* (9.01%) and *Lac. brevis* (3.60%) were different from Koki. The species are anaerobic and homofermentative which may also be responsible for the higher concentration of lactic acid production in Kom than Koki samples.

For instance, *Lac. paracasei* is the highest in population (90%) during final day of fermentation (day 45) when the pH of Kom was 4.10. In previous study by EFSA (2014), it was explained that these bacteria grow well in the pH range of 3.7–4.5, and survived as low pH as 1.5 to 2.5

(Edward and Farnworth, 2008). These investigations are consistent with the present study fermentation environment of Kom (pH = 4.10 and high acidity). The second dominant LAB species found in Kom sample was *Lac. brevis.* The strain grows in pH range of 4–4.5 and are homofermentative too (Gu, 2013; Edward and Farnworth, 2008; Liu and Dong, 2002).

Sahlin, (1999) and Stuart, (1986) reported that plant materials may be added to food processing to improve flavor, odor, taste, color and to increase safety, quality, and shelf-life of the foods. In the present study changes in *Kocho* composition observed in moisture contents, ash, protein, fiber, fat, water holding capacity, phytic acid contents and sensory attributes and many other parameters were observed as well.

The present study resulted in retention of moisture contents where it was almost constant throughout the fermentation period due to the fact that the fermentation was taken place in jars sealed by polyethene bags. In another study conducted by Gashe (1987a), where fermentation took place in fermentation pit, the moisture contents dropped from 84 to 60% within 0 to 79 days. According to Davey (1989), Frazier and Westoff (1978), a moisture content of any food is an index of its water activity and is used as a measure of stability and susceptibility to microbial contamination. In the present study, the high moisture contents of *Kocho* may be an indication of fermentation following the addition of *Mandillo*. However, there was less susceptibility to microbial spoilage of Kom than the Koki samples.

The protein and fat contents of *Kocho* are naturally very low as it was demonstrated by Yirmaga (2013), Tsegaye and Struik, (2001) and Gashe (1987a). The previous study by Aberoumand (2010) and Effiong *et al*. (2009) stated that any plant food that provides about 12% of its calories is valued as good source of protein. Therefore, foods from *Kocho* source in general need

supplementation with other rich source of protein and fat foods like egg, milk and legumes (Abebe *et al*., 2006); for example the Shekacho people who consume *Kocho* partly meet their protein and fat needs with animal and other plant protein source foods. This in turn can fight the problem of malnutrition in the study area. In this study, the maximum protein content of 3.95% (in Kom) showed better yield than that reported by Gashe (1987b) (0.5%), and less yield than by Yirmaga (2013) (5.09%). In this study the amount of fat evaluated was similar to the study by Keishing (2015). The amount of fat in both cases, in this finding were more than 2 folds of the content obtained in *Kocho* samples of previous work by Tsehaye and Kebebew (2006) who reported 0.21 to 0.30%. The disparities in protein and fat contents may be attributed to many factors such as differences in fermentation conditions, soil type, and geographical location of the fermented *Enset* plant, age and the local cultivar of the crop.

The carbohydrate content in the present work, compared to the previous study (45%) by Gashe (1987b) was remarkably high. The highest carbohydrate content of *Kocho* can also contribute to greater food security, since 45–65% of energy requirement of adult person per day is from carbohydrate (McGuire, 2010).

In the present study, the decrease in fiber contents as a result of fermentation period extension shared similar trend with the previous study (Yirmaga, 2013) who verified the decrement of fiber content from 2.67 to 1.89% within 20 days of fermentation time. The decrease in fiber content of *Kocho* during fermentation could also be attributed to the partial solubilisation or degradation of cellulosic and hemi-cellulosic structural materials in the plant (pulp) by microbial enzymes (Yirmaga, 2013). In lactic acid fermentation, *Lactobacillus plantarum* plays great role in the digestion of fiber (Cao, 2011).

According to Agostoni *et al*. (1995), adequate amount of crude fiber is responsible for the normal functioning of the intestinal tract. It increases stool bulk and decreases the time that waste materials spend in the gastrointestinal tract. Bulking fibers also absorb water as they move through the digestive system, easing defecation.

Fiber helps in the maintenance of human health and has been known to reduce cholesterol level of the body (Bello *et al*., 2008). A low fiber diet has been associated with heart disease, cancer of the colon and rectum, varicose veins, obesity, appendicitis, diabetes and constipation (Lajide *et al*., 2008; Saldanha, 1995). Even though the fiber content is highly decreased, still the contents in the study samples are greater than the daily adult requirement range of fiber content (25-38 g per day) (Zelman, 2014). Hence, *Kocho* could be recommended as a variable crude fiber source in the diet as a result of its relatively high fiber content.

The mineral contents of *Kocho* from Masha town is also incredibly high in quantity. A maximum of 3.40 mg/100g of Ca content in *Kocho* was reported by Zewdie (2008) while Atlabachew and Chandravanshi (2008) reported 58.47 mg/100g. As described in table 4.15, the result of Ca contents of the study area were about three times greater than that reported by Zewdie (2008) and six times less than that of Atilabachew and Chandravanshi (2008). These results also indicate that the mineral contents in *Kocho* may vary from place to place based on differences in age of the plant, soil type and fermentation techniques. For instance in the present study, there was no possibility of nutrient loss as a result of fermentation taking place in the jars sealed by polyethylene bags. The Ca content of *Kocho* in the present study area is also very high when compared to the Ca content of other starchy food source (Maize, 0.05%) (Assohoun, 2013). Zn and Cu contents of the present study *Kocho* samples are similar to the amount in brown rice (1.98 and 0.16 mg/100g), whereas Fe and Ca are higher than that of brown rice (0.57 and 6.85

mg/100g; respectively) (Heinemann, 2005). According to Soetan *et al*. (2010), minerals are vital for the overall mental and physical wellbeing and are important constituent of bones, teeth, tissues, muscles, blood and nerve cells.

As it was described by Frazier and Westoff (1978), the moisture content of any food is an index of its water activity. Like moisture content, water holding capacity is also used as a measure of stability and susceptibility to microbial contamination. In the present study, the difference in water holding capacity observed between Koki and Kom revealed the fact that Koki samples could be more susceptible to microbial contamination than Kom and less stable which may lead to short shelf-life compared to the experimental *Kocho* (Kom) samples. High moisture content foods have less energy that can be recommended for weight loss.

The decrease in phytic acid was more pronounced in Koki than Kom. Phytic acid digestion is facilitated by the activity of enzyme phytase, which works best around pH 5.5 (Singh et al., 2013). The pH of Koki was increased to 4.49 toward the final days of fermentation while that of Kom was lowered to 4.10. The decrease in phytic acid in Koki is also in line with the higher population of *Lac. plantarum* in the sample than in Kom, which produces phytase, the enzyme responsible for the digestion of phytic acid.

According to Assohoun *et al*. (2013) the mineral contents of substrates remained unaffected by fermentation process; however, availability increases as a result of minimized ant-nutrition contents in fermented foods, which is in agreement with the present study. The presence of phytic acid reduces the availability of zinc, manganese, copper, molybdenum, calcium, magnesium, iron as well as protein (Chanakan, 2006; Ferial and Georg, 2004). When bound to protein, phytic acid induces a decrease in solubility and functionality of the protein (Megat-

Rusydi, 2012). These processes can therefore contribute to iron and zinc deficiencies in people who rely only on high phytic acid content foods such as for their mineral intake, such as people in developing countries (Hurrell, 2003). The bioavailability of phytate phosphorus can be increased by supplementation of the diet with the enzyme phytase (Ali, 2010) or probiotic lactobacilli and other species of endogenous digestive microflora may be a sufficient source of the enzyme phytase to improve mineral absorption (Famularo, 2005).

In the present study, increase in fermentation days in Koki showed the highest reduction in tannin contents that may increase the availability of minerals in *Kocho* foods (Blandino *et al*., 2003; Henry, 2001). According to Rao *et al*. (1982), reduction of tannin to a low level, significantly increases in ionisable iron.

Conclusions

In the present study, total phenolic compounds, total flavonoids, antimicrobial activities and antioxidant capacities of *Mandillo* were evaluated to understand why *Mandillo* shows positive effect on *Enset* fermentation processes. The findings showed significant amounts of total phenol and flavonoids were present in *Mandillo*. These bioactive components may be responsible for antioxidant, antimicrobial and DNA protective activities seen in the results. Moreover, its selective anti-microbial activities revealed *Mandillo's* starter-culture role exhibited during *Enset* fermentation to produce good quality *Kocho* product. On the other hand, Mandillo selectively enhanced the growth of LAB which was more responsible to speed up the fermentation processes.

Likewise, microbial diversity in *Kocho* products in Ethiopia may not be identical in other situations. The results in the present study showed significant differences between the control and experimental *Kocho* samples. Even though the major species, *Lac. plantarum* is common to both Koki and Kom of *Kocho* samples, in the present study of *Enset* fermentation processes, some LAB and yeast species were unique in the experimental *Kocho* samples.

In this work, we evaluated the possibilities of *Mandillo* stem addition in *Enset* fermentation processes in increasing fermentation rate, enhancing self-life and improving organoleptic quality of *Kocho* product. The results obtained also strongly suggeste that the addition of *Mandillo* stem created favorable environment for useful microorganisms such as LAB species by suppressing (inhibiting) the growth of aerobic mesophile, coliforms, staphylococci and spore formers. *Mandillo* also enhanced species diversification of LAB strain which may produce diversified

bioactive ingredients that may contribute for the food safety, health and improved organoleptic quality of *Kocho*.

As a general conclusion, addition of *Mandillo* during *Enset* fermentation resulted in the improved products in all of the measured parameters towards favorable conditions to the food safety, quality and nutritionally improved products than the control groups.

Recommendations

Enset has untapped potential in ensuring food security in Ethiopia. The long years' of traditional experiences and practices of the Shekacho people in *kocho* fermentation should be maintained and extended to other *Enset* producing and none producing regions of the country.

As part of the outcomes of this study, the large differences observed between the female and male *Enset* types and the *Kocho* products, further research works related to DNA profiling of *Enset* is essential.

From these observations, it may be recommended that serious and detailed investigations shall be carried in the potential parts of the plant, to identify active components responsible for medicinal, food additive, food preservative, antimicrobial, antioxidant and DNA protective activities of *Mandillo*.

On top of these, the cytotoxicity of *Mandillo* stem was investigated. The ethanol extract of *Mandillo* showed very low cytotoxicity with 50% inhibitory concentration (IC_{50}) of 4 µg/mL against RAW 264.7 cells, suggesting that *Mandillo* stem may be a healthy choice and safe for starter culture ingredient to improve fermentation quality of *Kocho.* We also suggest that intensive research has to be conducted to use *Mandillo* stem in other food fermentation processing as supported by the work done by Yamada (1997).

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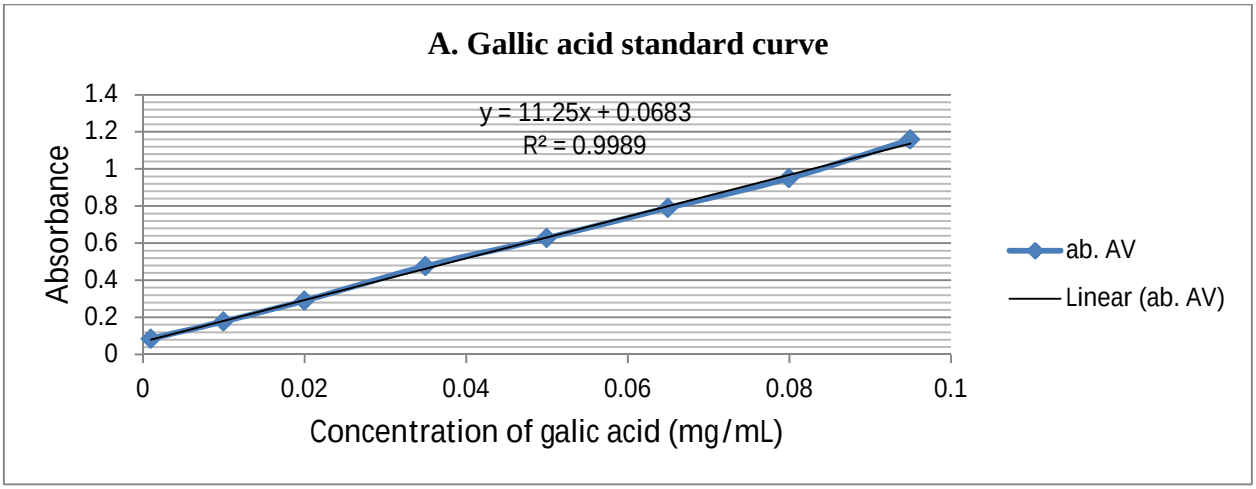
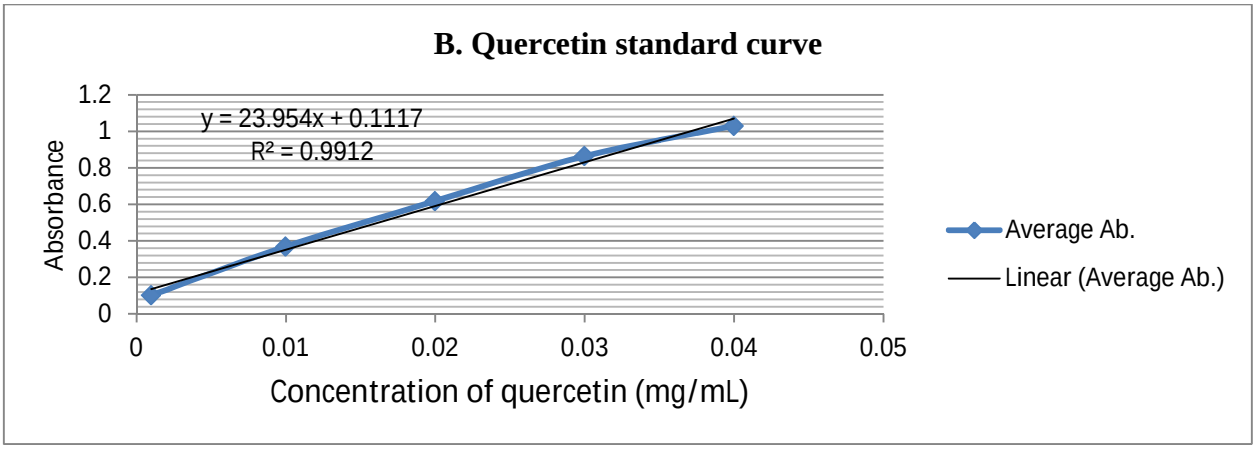
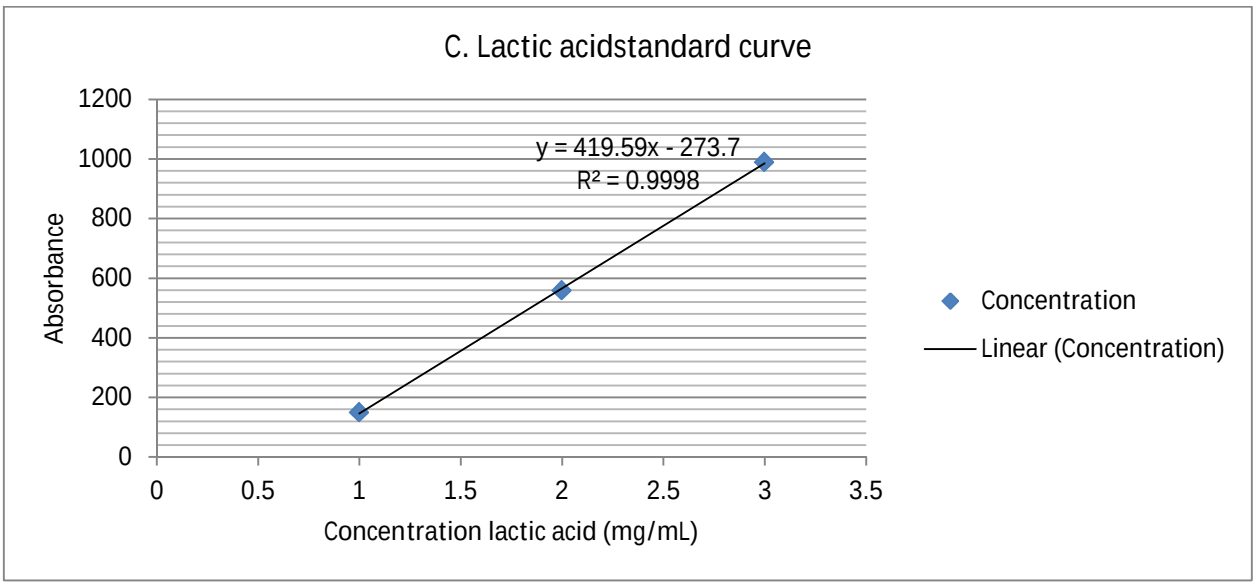
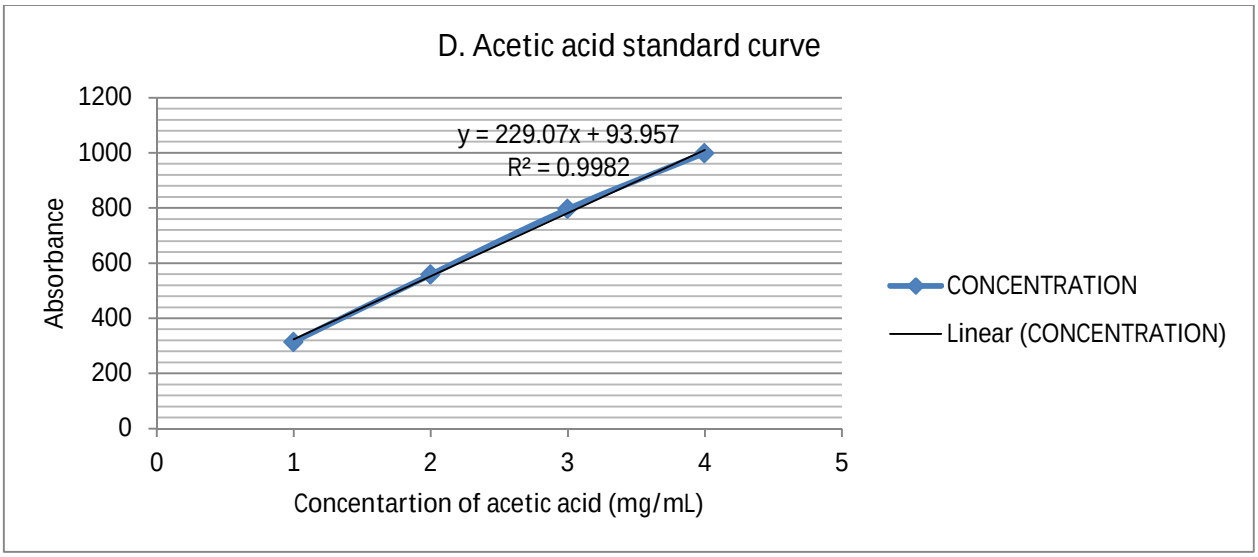
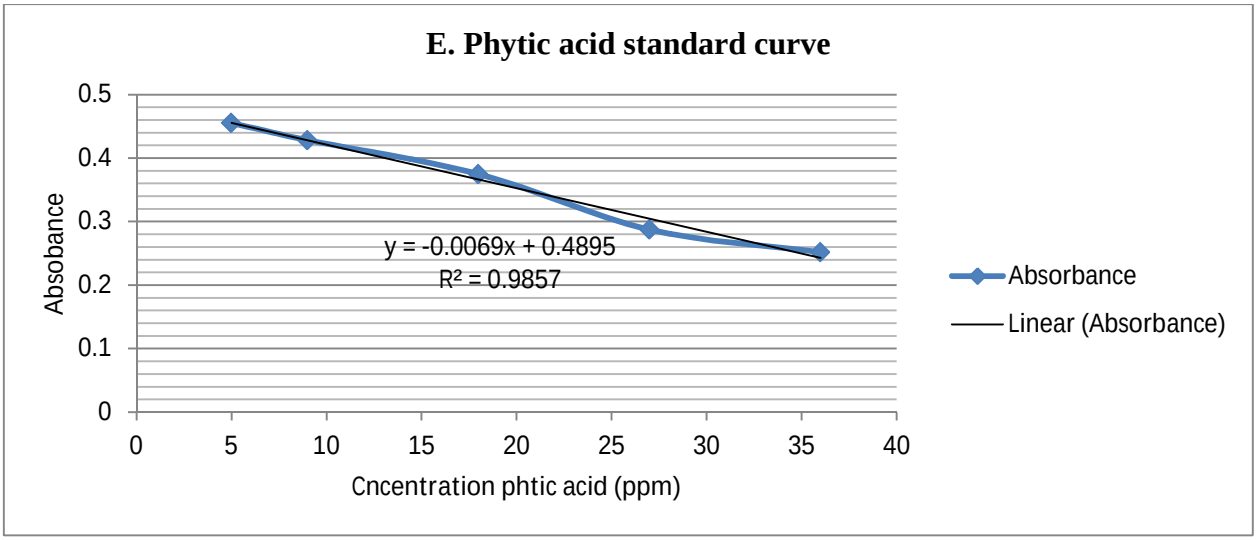
Appendix A: Standard curves of gallic acid (A) quercetin (B), lactic acid (C), acetic acid (D), phytic acid (E) and tannin (D-Catechin) (F)

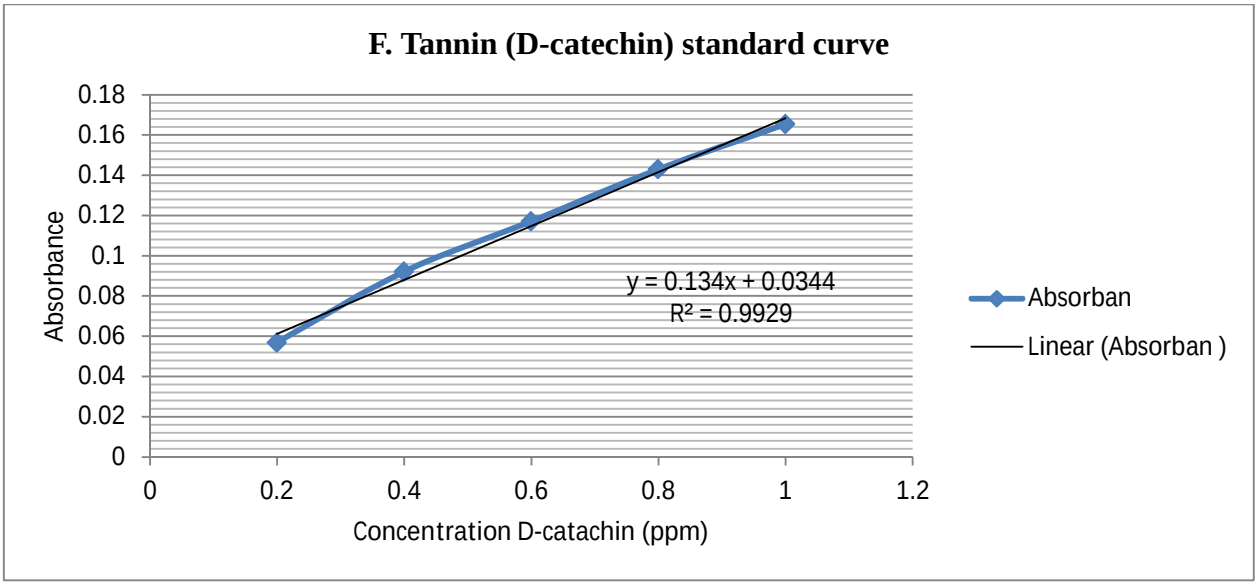







D. Acetic acid standard curve
y = 229.07x + 93.957
R² = 0.9982
Absorbance
Concentartion of acetic acid (mg/mL)
CONCENTRATION
Linear (CONCENTRATION)


E. Phytic acid standard curve
y = -0.0069x + 0.4895
R² = 0.9857
Absobance
Cncentration phtic acid (ppm)
Absorbance
Linear (Absorbance)


F. Tannin (D-catechin) standard curve
y = 0.134x + 0.0344
R² = 0.9929
Absorbance
Concentration D-catachin (ppm)
Absorban
Linear (Absorban)

Appendix B: Classification of yeast isolates using ribosomal DNA projects (RDP) Naïve, Bayesian rRNA Classifier, Version 2.10, with confidence threshold of 80%

Domain	Phylum	Class	Order	Family	Genus	Species	Groups name	No. isolates
	Phylum						*Ascomycota*	44
		Class					*Saccharomycetes*	44
			Order				*Saccharomycetales*	44
				Family			*Pichiaceae*	15
					Genus		*Pichia*	15
						Species	*Pichia fermentans*	8
					Unclassified		*Pichia*	7
				Family			*Incertae sedis* 11	23
					Genus		*Wickerhamomyces*	8
						Species	*Wickerhamomyces* sp ES22S03	8
					Genus		*Candida*	15
						Species	*Candida boidinii*	15
				Family			*Saccharomycetaceae*	6
					Genus		*Williopsis*	5
						Species	*Williopsis* sp	5
					Genus		*Kazachstania*	1
						Species	*Kazachstania exigua*	1
			Unclassified				Fungi	6

Appendix C: Sensory evaluation raw data

A. Sensory evaluation of *Kocho* pancake														
Code of panelists	**Texture**		**Color**		**Appearance**		**Odor**		**Flavor**		**Taste**		**Chewiness**	
	Koki	**Kom**	**Koki**	**Kom**	**Koki**	**Kom**	**Koki**	**Kom**	**Koki**	**Kom**	**Koki**	**Kom**	**Koki**	**Kom**
A01	**4**	5	**4**	5	**4**	5	**4**	5	**4**	5	**4**	5	**4**	5
A02	**3**	5	**3**	5	**3**	5	**3**	5	**3**	5	**5**	5	**3**	4
A03	**4**	5	**4**	5	**4**	5	**4**	5	**4**	5	**4**	5	**4**	5
A04	**4**	5	**4**	5	**3**	5	**3**	5	**4**	5	**4**	5	**3**	5
A05	**3**	5	**3**	5	**4**	5	**4**	5	**4**	5	**4**	5	**4**	5
A06	**3**	5	**3**	5	**3**	5	**4**	5	**3**	5	**5**	5	**3**	4
A07	**3**	5	**3**	5	**3**	5	**4**	5	**3**	5	**4**	5	**4**	5
A08	**3**	5	**3**	5	**4**	5	**4**	5	**3**	5	**5**	5	**4**	4
A09	**3**	5	**3**	5	**3**	5	**4**	5	**4**	5	**4**	5	**3**	4
A10	**3**	5	**3**	5	**3**	5	**4**	5	**4**	5	**4**	5	**4**	4
A11	**4**	5	**4**	5	**4**	5	**3**	5	**3**	5	**4**	5	**3**	4
A12	**4**	5	**4**	5	**3**	5	**4**	5	**4**	5	**4**	5	**4**	5
A13	**3**	5	**3**	5	**3**	5	**4**	5	**4**	5	**4**	5	**4**	4
A14	**3**	5	**3**	5	**4**	5	**4**	5	**3**	5	**4**	5	**3**	5
A15	**3**	5	**3**	5	**3**	5	**4**	5	**4**	5	**5**	5	**4**	4
A16	**3**	5	**3**	5	**4**	5	**3**	5	**4**	5	**4**	5	**3**	4
A17	**3**	5	**3**	5	**3**	5	**3**	5	**3**	5	**4**	5	**4**	5
A18	**3**	5	**3**	5	**3**	5	**3**	5	**4**	5	**4**	5	**4**	4
A19	**4**	5	**4**	5	**3**	5	**4**	5	**4**	5	**5**	5	**3**	4
A20	**3**	5	**3**	5	**3**	5	**3**	5	**3**	5	**5**	5	**4**	4
A21	**3**	5	**3**	5	**4**	5	**4**	5	**4**	5	**4**	5	**4**	4
A22	**4**	5	**4**	5	**3**	5	**3**	5	**4**	5	**4**	5	**4**	4
A23	**3**	5	**3**	5	**3**	5	**4**	5	**4**	5	**4**	5	**4**	4
A24	**4**	5	**4**	5	**3**	5	**4**	5	**3**	5	**4**	5	**3**	4
A25	**3**	5	**3**	5	**4**	5	**4**	5	**4**	5	**5**	5	**4**	4
A26	**3**	5	**3**	5	**3**	5	**4**	5	**4**	5	**4**	5	**4**	5
A27	**3**	5	**3**	5	**3**	5	**4**	5	**4**	5	**5**	5	**4**	4
A28	**3**	5	**3**	5	**3**	5	**3**	5	**4**	5	**5**	5	**4**	4
A29	**4**	5	**4**	5	**4**	5	**4**	5	**4**	5	**4**	5	**4**	4
A30	**4**	5	**4**	5	**3**	5	**3**	5	**3**	5	**5**	5	**3**	4
AV	3.33	5.00	3.33	5.00	3.33	5.00	3.67	5.00	3.67	5.00	4.33	5.00	3.67	4.33

B. Sensory evaluation of *Kocho* dough												
Code of panalists	**Texture**		**Color**		**Appearance**		**Odor**		**Flavor**		**Taste**	
	Koki	**Kom**	**Koki**	**Kom**	**Koki**	**Kom**	**Koki**	**Kom**	**Koki**	**Kom**	**Koki**	**Kom**
A01	**4**	5	**4**	5	**4**	5	**4**	5	**4**	5	**4**	4
A02	**3**	5	**3**	5	**4**	5	**3**	5	**3**	5	**3**	4

A03	**4**	5	**4**	5	**4**	4	**4**	5	**4**	5	**4**	4
A04	**4**	5	**4**	5	**4**	4	**3**	5	**4**	5	**4**	4
A05	**3**	5	**3**	5	**4**	4	**3**	5	**4**	5	**4**	4
A06	**3**	5	**3**	5	**4**	5	**3**	5	**3**	5	**3**	4
A07	**3**	5	**3**	5	**4**	4	**4**	5	**3**	5	**4**	4
A08	**3**	5	**3**	5	**4**	4	**3**	5	**3**	5	**3**	4
A09	**3**	5	**3**	5	**4**	5	**4**	5	**4**	5	**4**	4
A10	**3**	5	**3**	5	**4**	4	**3**	5	**4**	5	**4**	4
A11	**4**	5	**4**	5	**4**	5	**3**	5	**3**	5	**3**	4
A12	**4**	5	**4**	5	**4**	4	**4**	5	**4**	5	**4**	4
A13	**3**	5	**3**	5	**4**	5	**3**	5	**4**	5	**3**	4
A14	**3**	5	**3**	5	**4**	5	**4**	5	**3**	5	**4**	4
A15	**3**	5	**3**	5	**4**	4	**4**	5	**4**	5	**3**	4
A16	**3**	5	**3**	5	**4**	4	**3**	5	**4**	5	**4**	4
A17	**3**	5	**3**	5	**4**	4	**3**	5	**3**	5	**3**	4
A18	**3**	5	**3**	5	**4**	4	**3**	5	**4**	5	**4**	4
A19	**4**	5	**4**	5	**4**	4	**4**	5	**4**	5	**4**	4
A20	**3**	5	**3**	5	**4**	4	**3**	5	**3**	5	**3**	4
A21	**3**	5	**3**	5	**4**	5	**3**	5	**4**	5	**4**	4
A22	**4**	5	**4**	5	**4**	5	**3**	5	**4**	5	**4**	4
A23	**3**	5	**3**	5	**4**	4	**3**	5	**4**	5	**4**	4
A24	**4**	5	**4**	5	**4**	4	**3**	5	**3**	5	**4**	4
A25	**3**	5	**3**	5	**4**	4	**4**	5	**4**	5	**4**	4
A26	**3**	5	**3**	5	**4**	4	**3**	5	**4**	5	**4**	4
A27	**3**	5	**3**	5	**4**	4	**3**	5	**4**	5	**4**	4
A28	**3**	5	**3**	5	**4**	5	**4**	5	**4**	5	**3**	4
A29	**4**	5	**4**	5	**4**	4	**3**	5	**4**	5	**4**	4
A30	**4**	5	**4**	5	**4**	4	**3**	5	**3**	5	**3**	4
AV	3.33	5.00	3.33	5.00	4.00	4.33	3.33	5.00	3.67	5.00	3.67	4.00

Appendix D: Questionnaire paper for the study of indigenous knowledge of the Shekacho society (Yemane and Fassil, 2006)

Addis Ababa University
School of Graduate Studies
Center for Food Science and Nutrition

PhD Research Questionnaire Paper

Title of the PhD Research: ***Synergetic effects of Mandillo during Ensete Fermentation: Chemical Nature and Efficiency***

Title of objective one: ***Indigenous knowledge of Sheka society of Enset cultivation, fermentation and food preparation***

Objective: to investigate the indigenous knowledge of the Shekacho society on ***Enset*** plantation, fermentation processes and *Kocho* food preparation

The Significance of the questionnaire is to:

- investigate the indigenous/local practices of ***Enset*** fermentation processes in Sheka society
- apply the findings in the research work of the PhD proposal
- publicize the best practices of the society on ***Enset*** plantation, fermentation processes and *Kocho* food preparation

We appreciate your response. All of the answers you provide in this survey will be kept confidential. No identifying information will be provided to any party. The survey data will be reported as a summary only and will not identify any individual person. Fifty focused individuals will be selected. The focus groups are growers 30%, consumers 30% and professionals 40% of age 18 and above and both sex groups. The respondents will be selected purposefully to find the most knowledgeable individuals among the society. Descriptive analysis of data will take place.

Thank you for participating in the PhD study information gathering activities. If you have any questions regarding the feedback of this questionnaire please contact *Mr. Alemu Gonfa Robi* by phone *+251-911397348* or email *alemug4@gmail.com* *or face to face.*

I. Personal particulars

Name: ……**Sex:** Male ☐ Female ☐ **Age:** 18-20 years ☐ 20-30 years ☐ 30-50 years ☐ > 50 years ☐

Occupation: Teacher ☐ Farmer ☐ Agricultural expert ☐ Student ☐ (grade/level……..) experienced person ☐ Specify: ……

Relation to *Enset*: Consumer ☐ Grower ☐ Researcher ☐ Specify: ……

For how long you are associated to *Enset*: < 10 years ☐ 18-30 years ☐ 30-50 years ☐ > 50 years ☐ ……

II. History of *Enset*

1. For how long do you think is ***Enset*** known in Sheka Zone? 100 years ☐ 500 years ☐ 1000 years ☐ More than 1000 years ☐ Unknown ☐ It is as long as the history of Sheka Society ☐
Specify: ……

2. In what ways were ***Enset*** plant used? Mark by "X" in the following table

Parts of Enset plant/products	Food	Animal feed	Medicine	Household utensils	Construction materials	Detergent	Clothes			
							Dubbo	Qocho	Gichoo	Fussoo
Leaf/Maato										
Leafsheath/Yo'o										
Squeezed liquid/Quuroo										
Midrib/Ooqqo										
Pseudo-stem parts/Dubo										
Corm/Uuxo										
Kocho										
Bula/Ittino										
Starter culture/Kiisho										
Fiber/Yi'o										

Specify: …….

3. Where do you think is the origin of Sheka Zone ***Enset***? SNNPR ☐ Oromia ☐ Binishangul-Gumuz ☐ Africa ☐ Since our forefathers Enset is found in Sheka Zone ☐ Specify: ……

4. Do you know if the Sheka Zone ***Enset*** was taken to other regions? Yes ☐ No ☐

5. If your answer in question 4 is **yes**, indicate the regions: SNNPR ☐ Oromia ☐ Binishangul-Gumuz ☐ Amahara ☐ Tigray ☐ Africa ☐ Specify: ……

6. Have you ever experienced starvation in the history of Sheka Zone society? Yes ☐ No ☐

7. If your answer in question 6 above is **yes** how often it was happening? Every year ☐ Once in four years ☐ Once in a decade ☐ Once in a century ☐ Unknown ☐ Specify ……

8. If your answer in question 6 above is **no** why it was not happening? Because of Enset plant ☐ Because of fertile land ☐ Because of forest ☐ Unknown ☐ Specify ……

9. Have you ever experienced drought in Sheka Zone? Yes ☐ No ☐

10. If your answer in question 9 above is **yes** how often it was happening? Every year ☐ Once in four years ☐ Once in a decade ☐ Once in a century ☐ Not happening ☐ Specify ……

11. If your answer in question 9 above is **no** why it was not happening? Because of Enset plant ☐ Because of the altitud ☐ e Because of forest ☐ Unknown ☐ Specify ……

III. Cultivars of *Enset* in Sheka Zone

12. How many Cultivars of ***Enset*** are found in Sheka Zone? …….

13. Indicate the name and the type of the varieties of ***Enset*** found in Sheka Zone marking by "**X**" and F (female) or M (male) respectively

Enset variety	Name	F or M	Enset variety	Name	F or M	Enset variety	Name	F or M	Enset variety	Name	F or M
1											
2											
3											
…N											

Specify……

14. Which varieties are most commonly available? (Rate as 1st , 2nd , 3rd, …….)

Enset variety	Rate	Enset variety	Rate	Enset variety	Rate	Enset variety	Rate	Enset variety	Rate
1									
2									
3									
…N									

Specify:……

15. What are the ways/ methods of identification of ***Enset*** plant varieties? Leaf-color ☐ Structure of leaf ☐ Size of ***Enset*** plant ☐ Pseudo-stem color ☐ Structure of pseudo-stem ☐ Size of leaf ☐ Size of pseudo-stem ☐ Midrib color ☐ Petiole color ☐ Specify: ……

16. What are common colors and structure of ***Enset*** plant parts of Sheka Zone? *(Yemane T. et.al, 2006)*

a. **Leaf color (LC):** Light green ☐ Deep green ☐ Light red ☐ Dark red ☐ Purple ☐
Specify: ……

b. **Midrib color (MC):** Light green ☐ Deep green ☐ Greenish yellow ☐ Greenish red ☐ Light red ☐ Dark red ☐ Dark brown ☐ Specify: …….

c. **Petiole color (PC):** Light green ☐ Deep green ☐ Yellowish green ☐ Light red ☐ Ddark red ☐ Reddish yellow ☐ Specify: ……

d. **Pseudostem color (PSC):** Light green ☐ Deep green ☐ Greenish black ☐ Light red ☐ Dark red ☐ Reddish yellow ☐ Dark brown ☐ Purple ☐ Yellowish green ☐ Dark red at one side and yellowish grey in the other ☐ Light red at the base ☐ Brownish yellow above the base ☐ Deep green above the base ☐ Specify: ……..

e. **Pigmentation (PT):** None ☐ Light red stripe ☐ Light red patches ☐ Dark red spots ☐ Dark brown patches ☐ Green stripe ☐ Green patches ☐ Greenish yellow patches ☐ Greenish yellow stripes ☐ Yellowish grey stripe ☐ Specify: …….

f. **Corrugation (CR):** None ☐ Slightly corrugated ☐ Highly corrugate ☐ Specify: …… ……

17. List some of the ***Enset*** varieties based on food and fiber quality and quantity, medicinal value, disease and drought resistance

No	***Quantity food***			***Quality food***			***Medicinal value***	***Drought resistant***	***Disease resistant***
	Kocho	***Bula***	***Fiber***	***Kocho***	***Bula***	***Fiber***			
1									
2									
3									
…									
N									

18. Mark as **"X"** the type of Enset plant that have more of the following properties

Type	***Quantity of food***			***Quality of food***			***Medicinal value***	***Drought resistant***	***Disease resistant***
	Kocho	***Bula***	***Fiber***	***Kocho***	***Bula***	***Fiber***			
Male									
Female									

19. Which type of disease and wild life affect ***Enset*** plant most? Bacterial wilt/Kiino ☐ Buunno ☐ *Enset* root mealybug ☐ Fungi ☐ Mole rats ☐ Porcupine ☐ Wild pig ☐ Monkeys ☐ Specify …… ……

20. In which age of its growth does ***Enset*** plant susceptible to disease? One year ☐ Two years ☐ Three years ☐ Four years ☐ Five years ☐ All year ☐ Specify ……

21. Which part of the ***Enset*** plant would be affected by the disease? The leaf ☐ The root ☐ The pseudo-stem ☐ The corm ☐ All parts ☐ Specify ……..

22. In the following table, indicate which part of Enset plant can be affected by which disease or/and wildlife marking by **"X"**

Parts of Enset	Kiino	Buunno	Fungi	Mealybug	Mole rats	Porcupine	Wild pig	Monkeys
Leaf								
Root								
Pseudo-stem								
Corm								
All parts								

IV. Medicinal values of *Enset*

23. The *Enset* plant and its products are medicine for: Human being ☐ Domestic animals ☐ Wild life ☐ Specify …….

24. The *Enset* plant and its products are important to cure: Broken bones ☐ Fractures joint displacement ☐ Swelling with pus ☐ Stimulates the placental discharge ☐ Abortifacient ☐ Diarrhea ☐ Infectious disease ☐ Specify ……

25. Which parts/products of ***Enset*** plant have medicinal value for the indicated disease? Mark by **"X"**

Parts of Enset	Broken bones	Fractures joint displacement	Swelling with pus	Stimulates the placental discharge	Abortifacient	Diarrhea	Infectious disease
Leaf							
Squeezed liquid							
Pseudo-stem							
Corm							
Kocho							
Bula							
Starter culture							
Fiber							

V. Cultivation of *Enset*

26. Who is responsible in ***Enset*** cultivation activities? The male ☐ The female ☐ Both ☐

27. Describe the methods and stages of development of ***Enset*** cultivation by marking an "X" in the following table

Methods/processes	Stages of development in month (m) or year (yr)									
	For male after					For female after				
	3-6 m	6-12m	1-2yr	4-5yr	Specify	3-6m	6-12m	1-2yr	3-4yr	Specify
Removal of the pseudostem from the corm/Uuxe maacco										
Division of the corm in to 2-4 parts/Baqqo										
Putting the corm in a pit upside down/Duukko										
Or left intact to the ground coated with cow dung/Ibe-fuutto										
Sucker formation/Gaammo										
Seedling dispersion/aco										
Further separation of the seedling/Uukko										
Final transplantation of the seedling/Tokko										
Total age to harvest										

28. How many times ***Enset*** would be transplanted before maturation? Once ☐ Twice ☐ Three times ☐ Specify …….....

29. What type of soil is important in ***Enset*** cultivation? Red soil ☐ Clay soil ☐ Red-brown soil ☐ Loom soil ☐ Specify …… … … …….

30. Which altitude is favorable for ***Enset*** cultivation? 1500-1800 m ☐ 1800-2000 m ☐ 2000-2200 m ☐ 2200-2500 m ☐ 2500-3000 m ☐/ Kola ☐ Weinadega ☐ Dega ☐ Specify …….....

31. What type of rainfall is favorable for ***Enset*** cultivation? Low ☐ Medium ☐ High ☐ Heavy ☐ Specify …….....

32. What are the ranges of altitudes and rain fall in ***Enset*** cultivating areas of Sheka Zone? ……and …….respectively

33. What should be the distance between each ***Enset*** plant?

a. For female: 1.0 m ☐ 1.5 m ☐ 2.00 m ☐ 2.5 m ☐ Specify …….....

b. For male: 1.0 m ☐ 1.5 m ☐ 2.00 m ☐ 2.5 m ☐ Specify …….....

34. How long does ***Enset*** plant keep on without water?

a. The male: One year ☐ Two years ☐ Three years ☐ Four years ☐ Five years ☐ Specify …….....

b. The female: One year ☐ Two years ☐ Three years ☐ Four years ☐ Five years ☐ Specify …….....

35. What is the average height of commonly cultivated ***Enset*** plant? ……..

36. What is the average length of the commonly cultivated Enset leaf? ……

37. What is the average width of the commonly cultivated Enset leaf? ……

38. What is the average height of commonly cultivated ***Enset*** pseudo-stem? ……

39. What is the average circumference of commonly cultivated ***Enset*** pseudo-stem? ……

40. What is the average volume of commonly cultivated ***Enset*** plant corm? ……

41. What is the average weight of commonly cultivated ***Enset*** plant corm? …… ……

42. What is the average weight of commonly cultivated ***Enset*** plant pseudo-stem? ……

43. What is the average Kilogram of Kocho, Bula and Fiber obtained from one matured and commonly cultivated ***Enset*** plant respectively?

a. Male:……., …… and…….. respectively
b. Female:……., …… and…….. respectively

VI. Enset **fermentation processes**

44. Who is most responsible in ***Enset*** fermentation processes? The male ☐ The female ☐ Both ☐

45. How long ***Enset*** plant would stay before harvesting?

a. For the male type: Three years ☐ Four years ☐ Five years ☐ Six years ☐ Seven years ☐ Specify …….....

b. For the female type: Three years ☐ Four years ☐ Five years ☐ Six years ☐ Seven years ☐ Specify …….....

46. How is *Kocho* produced from ***Enset*** plant?
……

47. How can Bula/Etino be obtained from ***Enset*** plant?
……

48. What are the important preconditions/items to be prepared before starting ***Enset*** fermentation processes? Selection of mature Enset ☐ Site clearance ☐ Digging of the pit or preparation of place/Gamoo ☐ Preparation of starter culture/Kiisho ☐ Preparation of Enset leaf and heat on fire ☐ Preparation of clothes made up of Enset leaf ☐ Collection of different utensils useful in the processes ☐ Sharpening tools like knife ☐ Specify ……
……

49. What are the materials/equipments/utensils used in ***Enset*** fermentation processes? Wooden board/ Matoo ☐ Bamboo scraper/Maaro ☐ Kumixoo ☐ Bucket ☐ Specify……
……..

50. What are the procedures that can be followed in ***Enset*** fermentation processes? Removal of all leaves (*Maato*) and dried leafs-sheaths(Kaakiro) ☐ Removal of the interlocking leaf-sheaths ☐ Scraping of the

pseudostem ☐ Pulverization of the corm ☐ Chopping of the resulting biomass ☐ Grating the starter culture to the biomass and mixing ☐ Sealing off the resulting biomass from the external air with dried leaf sheaths ☐ Squeezing fermented *ensete* biomass ☐ Specify ……..

51. How many different products are obtained from ***Enset*** plant?.....What are they? The leaf/Maato ☐ The leaf sheath/Yo'o ☐ The squeezed liquid/Quuro ☐ The midrib/Ooqqo ☐ The Petiole/Qetto ☐ The starter culture/Kiisho ☐ The Eekko ☐ The Bacco ☐ The Corm or amicho/Uuxo or Ko'o ☐ The Kocho ☐ The Bula/Ittino ☐ The fiber/yi'o ☐

52. Which seasons are favorable for ***Enset*** fermentation processes? Kiremt ☐ Tsedey ☐ Bega ☐ Belg ☐ Specify …… …

53. After how many days will the scraped ***Enset*** biomass be put into a pit? One ☐ Two ☐ Three ☐ Four ☐ Specify……

54. What happen if the biomasses are not put in a pit? It is not fermented ☐ It is spoiled ☐ Unknown ☐ specify …… ……

55. What is the interval/day for Kocho to be taken off from pit to expose to the external air and remix the biomass?

a. If starter culture is already there:

- For female Enset: Four days ☐ Five days ☐ Six days ☐ Seven days ☐ Eight days ☐ Nine days ☐ Specify ……..
- For male Enset: Four days ☐ Five days ☐ Six days ☐ Seven days ☐ Eight days ☐ Nine days ☐ Specify ……..

b. If the starter culture is not ready

- For female Enset: Four days ☐ Five days ☐ Six days ☐ Seven days ☐ Eight days ☐ Nine days ☐ Ten days ☐ Twelve days ☐ Fourteen days ☐ Specify…… …….
- For male Enset: Four days ☐ Five days ☐ Six days ☐ Seven days ☐ Eight days ☐ Nine days ☐ Ten days ☐ Twelve days ☐ Fourteen days ☐ Specify…… …….

56. Why the biomass is exposed to the external air? …….
……

57. How many days does it take for *Kocho* to be ready for consumption during fermentation?

a. If starter culture is already there:

- For female Enset: Specify ……..

- For male Enset: Specify ……..

b. If the starter culture is not ready

- For female Enset: Specify…… …….
- For male Enset: Specify…… …….

58. What are the differences between?

a. *Bulla*: Fermented ☐ Unfermented ☐ Fiber free ☐ With fiber ☐ Mixed with other products ☐ Specify …….. …….

b. *Kocho*: Fermented ☐ Unfermented ☐ Fiber free ☐ With fiber ☐ Mixed with other products ☐ Specify …….. ……

59. What is the shelf-life of *Kocho* dough after fermentation? One month ☐ Two months ☐ Three months ☐ Six months ☐ Nine months ☐ One year ☐ Specify ……

60. What is the shelf-life of *Bulla* after production? One month ☐ Two months ☐ Three months ☐ Six months ☐ Nine months ☐ One year ☐ Specify ……..

61. What are the factors that affect the shelf-life of *Kocho* and *Bulla*? Air ☐ Moisture ☐ Hygiene problem ☐ Quality of packaging materials ☐ The presence or the absence of preservatives ☐ Specify…… ……..

62. Which one do you think is the best product? *Bulla*/Ittino ☐ kocho ☐ Eekko ☐ Amicho/Uuxo ☐ Bachoo ☐ Spesify……

63. How do you protect *Bulla* and *Kocho* from spoilage? By air tight ☐ By keeping away from moisture ☐ Good hygiene ☐ By good quality of packaging materials ☐ By adding traditional presence ☐ Specify…… ……..

64. What are the traditional preservatives of *Kocho* and *Bulla*? *Kocho* ☐ Shoobbo ☐ Kefo ☐ Toocho ☐ Duqqishe-Waawo ☐ Specify ……

65. How do you know whether *Kocho* and *Bulla* are spoiled or not? Off odor ☐ Off color ☐ Off taste ☐ Structure of dough ☐ Palatability ☐ Specify……

66. ***Enset*** fiber is used to prepare: Uuwo ☐ Dabbo ☐ Wadaro ☐ Fusso ☐ Dubbo ☐ Axxillo ☐ Marawo ☐ Gaawo ☐ Shuya ☐ Kocho ☐ Specify……

VII. Preparation of starter culture

67. Do you know that starter culture is required in ***Enset*** fermentation processes? Yes ☐ No ☐

68. If your answer in question 68 is **yes** which part/product of ***Enset*** is used for starter culture/Kiisho preparation? Shito ☐ Bakko ☐ Ooqqo ☐ Qetto ☐ Quuro ☐ Uuxo ☐ Specify……

69. What are the additional materials/herbs from which starter culture is prepared for ***Enset*** fermentation? Mandilo ☐ Huppisso ☐ Giraawo ☐ Yaabbagaawi ☐ Specify……

70. What are the methods used for the preparation of starter culture in ***Enset*** fermentation processes? Preparation of the bowel shaped container (Kishe goono) from the corm intact to the ground ☐ Addition of Bakko, Ooqqo, Shito, Quuro and Mandilo to the bowel ☐ Chopping and mixing well the ingridients ☐ Sealing off the starter culture from the external air with dried leaf sheaths ☐ Speciefy ……

71. How many days does the starter culture take to be ready for use in ***Enset*** fermentation?

- For female Enset Four days ☐ Five days ☐ Six days ☐ Seven days ☐ Eight days ☐ Nine days ☐ Ten days ☐ Twelve days ☐ Fourteen days ☐ Specify…… ……..
- For male Enset Four days ☐ Five days ☐ Six days ☐ Seven days ☐ Eight days ☐ Nine days ☐ Ten days ☐ Twelve days ☐ Fourteen days ☐ Specify…… ……

72. What are the uses of the starter culture? To improve the color of dough ☐ To improve the odor of dough ☐ To improve the flavor of dough ☐ To improve the taste of bread ☐ To improve the fermentation rate ☐ To increase the shelf-life ☐ Specify…… …… ……

73. Which of the herbs is the most important to prepare the starter culture? Mandilo ☐ Huppisso ☐ Giraawo ☐ Yaaba-gaawi ☐ Specify……

VIII. Uses of *Mandilo* in *Enset* fermentation processes

74. What are the uses of ***Mandilo*** in ***Enset*** fermentation processes? To improve the color of dough ☐ To improve the odor of dough ☐ To improve the flavor of dough ☐ To improve the taste of bread ☐ To improve the fermentation rate ☐ To increase the shelf-life ☐ Specify……
…… …… … ….. …

75. Which parts of ***Mandilo*** are important in preparation of the starter culture? The root ☐ The stem ☐ The leaf ☐ The flower ☐ Specify ……..……

76. In which form ***Mandilo*** applied to the starter culture? Chopped ☐ Powdered ☐ As it is ☐ Specify …… ……..

77. If ***Mandilo*** is not used, what would happen to the final product of ***Enset*** fermentation? ……

78. Does ***Mandilo*** have other uses than the aforementioned applications? Yes ☐ No ☐

79. If your answer in question 75 is **yes**, please list them…….. ……

80. How is ***Mandilo*** cultivated? …….. ……

81. Which season is the best to cultivate ***Mandilo***? Kiremt ☐ Tsedey ☐ Bega ☐ Belg ☐ Specify …… …… …

***IX. Enset* food preparations**

82. Who is responsible in the preparation of Kocho and Bula foods? The male ☐ The female ☐ Both ☐

83. How often do you eat Kocho or Bula food? Once a day ☐ Twice a day ☐ With all meals of the day ☐ Weekly ☐ Unknown ☐ Specify ……

84. Kocho and Bula foods are consumed with? Egg ☐ Kale ☐ Meat ☐ Milk products ☐ Legums ☐ Specify …… …… …. …….

85. Why do you consume Kocho and Bula? I have no options ☐ It is my favorite food ☐ As a variety food ☐ Specify …… … …… ……

86. What are some of food products prepared from:

a. From Kocho? Pancake/Maxino ☐ Porridge/Buuxo ☐ Dumpling/Kosso ☐ Enjera ☐ Xesso ☐ Naakasho ☐ Xappo ☐ Baacuuroo ☐ Kujjo ☐ Qaafoo ☐ Qoriyo ☐ Anjaawuro ☐ Specif …… …….. ……

b. From Bula? Pancake Porridge Dumpling Enjera Pancake/Maxino ☐ Porridge/Buuxo ☐ Dumpling/Kosso ☐ Enjera ☐ Xesso ☐ Naakasho ☐ Xappo ☐ Baacuuroo ☐ Kujjo ☐ Qaafoo ☐ Qoriyo ☐ Anjaawuro ☐ Specify …… ……

c. From Eekko? Pancake Porridge Dumpling Enjera Pancake/Maxino ☐ Porridge/Buuxo ☐ Dumpling/Kosso ☐ Enjera ☐ Xesso ☐ Naakasho ☐ Xappo ☐ Baacuuroo ☐ Kujjo ☐ Qaafoo ☐ Qoriyo ☐ Anjaawuro ☐ Specify …… ……

d. From Amicho/Uuxoo …….

87. Write the processes of preparation of foods:

a. From Kocho ……
……
……

b. From Bula ……
……

88. What is the shelf-life of food prepared from?

a. Kocho: One day ☐ Two days ☐ Three days ☐ One week ☐ Two weeks ☐ Three weeks ☐ One month ☐ Specify…… … …….

b. Bula: One day ☐ Two days ☐ Three days ☐ One week ☐ Two weeks ☐ Three weeks ☐ One month ☐ Specify…… … …….

c. Eekko: One day ☐ Two days ☐ Three days ☐ One week ☐ Two weeks ☐ Three weeks ☐ One month ☐ Specify…… … …….

89. Do you know techniques that are important to increase the shelf-life of food prepared by ***Enset*** sources?

Yes ☐ No ☐

90. If your answer in quastion 90 is **yes**, What are the techniques that can be employed ? By air tight ☐ By keeping away from moisture ☐ Good hygiene ☐ By good quality of packaging materials ☐ By adding traditional preservatives ☐ Specify……
……

91. If you have additional ideas? Please use the back page:

Thank You!