



ADDIS ABABA UNIVERSITY
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, CELLULAR AND MOLECULAR BIOLOGY
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Microbiological and Physicochemical Study of *Azo*, A Traditional Fermented Condiment Prepared from Sorghum and Leaves of Endod (*Phytolacca dodecandra*) in Kafta Humera, Tigray Regional State.

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DECLARATION

I, the undersigned, declare that the thesis entitled “Microbiological and Physiochemical Study of Azo, A Traditional Fermented Condiment Prepared from Sorghum and Leaves of Endod (*Phytolacca dodecandra*) in Kafta Humera, Tigray Regional State” is my original work and I did not present it for other degree in any university or institution.

Letay Gebrelibanos

Signature _____

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ACRONYMS AND ABBREVIATIONS

AMB= Aerobic Mesophilic Bacteria

ANOVA= Analysis Of Variance

AOAC= Association of Official Analytical Chemists

AZH=Azo from House hold

AZL= Azo from Laboratory

CFU= Colony Forming Unites

FC= Fecal Coliforms

LA= Lactic acid

MRS=De Man, Rogosa and Sharpe

PCA= Plate Count Agar

PDA= Potato Deoxtrose Agar

TA =Titratable Acidity

TC= Total Coliforms

VRBA= Violate Rad Bail Agar

XLDA=Xylems Lysine Deoxycholate Agar

TSA= Trypticase soy agar

ABSTRACT

Azo is a traditional fermented condiment prepared from cereal flour and leaves of endod in northwest Ethiopia Tigray regional state, particularly Kafta Hmera. The fermentation process is carried out spontaneously in traditional smoked container calabash or earthen pot. The objective of this study was to examine the physicochemical and microbial feature of Azo and identifies the predominant microorganisms those carried out fermentation. To this end, four ready to consume azo samples were collected from different households of Kafta after 6 days of fermentation and one sample was prepared in the laboratory. Samplings for laboratory prepared azo were taken every 24 hours interval for seven days; other two samples were taken from fifteen, and thirty days of fermentation to study the physicochemical and microbial profiles during fermentation. Accordingly, the pH was drastically dropped from 6.58 to 3.75; with 6-fold increase in titratable acidity (TA); with progressive increase in water content (from 72.23 to 80.60%); protein (from 3.28±0.04 to 4.87%). The result also showed that decrease in fat (from 4.19 to 3.75%), carbohydrate (from 17.45 to 8.6%) and ash content (from 2.86 to 2.11%) from 0 to 6 days of fermentation. At the first six to seven days of fermentation sharp increase in lactic acid bacteria (LAB) from 3.35 to 10.49 and 3.68 and aerobic mesophilic bacteria (AMB) count from 3.68 to 10.13 log of CFU/g were observed. Whereas, yeast increased slightly up to second day of fermentation and after 5th day of fermentation they were

disappeared. The total coliforms (TC) and faecal coliforms (FC) were only detected at first two and three days of fermentation and Salmonella was not detected throughout the fermentation period. Based on morphological, physiological and some biochemical tests, a total of 42 LAB from azo were identified into five genera; Lactobacillus (52%), Pediococcus (21%) Lactococcus (10%), Leuconostoc (10%), Enterococcus (5%) and one unidentified genus (2%). In terms of occurrence throughout the fermentation, Lactobacillus and Pediococcus were the dominant lactic acid bacteria. Based on the results it can be concluded that fermentation shows good improvement in cured protein content and microbial safety of the product.

Keywords/phrases: Azo, Fermentation, Homofermentative lactic acid bacteria, Kafta, Microbial load, Protein content, titratable acidity,

1. INTRODUCTION

Humankind has consumed fermented foods since time immemorial (Subhashree and Patel, 2013). Fermentation is one of the widely practiced ancient technologies and used to prepare essential part of diets with long history among different culture in all regions of the world. The process serves as a mean of providing a source of nourishment for large rural populations that enhances the physicochemical composition and nutrient content of foods through the synthesis of proteins, vitamin, and essential amino acids (Zhang *et al.*, 2010) by the action of microorganisms.

Fermentation also plays an important role in ensuring the food security of millions of people around the world, particularly marginalized and vulnerable groups (Marshall and Mejie, 2011). It also leads to detoxification, destruction of undesirable factors present in raw food. In general, it is oldest and economic methods of preserving the quality and safety of foods (Rhee *et al.*, 2011).

It estimated that 25% of the European diet and 60% of the diet in many developing countries consist of fermented foods (Stiles, 1996; Ogunbanwo *et al.*, 2004). In Ethiopia, fermented foods have been known since time of immemorial. According to Dirar (1993), Ethiopians had been brewing Merissa like beer since 7 BC. In addition, Ethiopia is known for its cultural and traditional heterogeneity; they have different ethnic backgrounds and their own fermented food and beverage (Mogessie Ashenafi, 2006). In their production, microorganisms play a vital and essential role contributing to the improvement of the physicochemical, sensory and safety characteristics of the final products (Esayas Assefa *et al.*, 2008).

Among the diversified traditional fermented foods currently mentioned are those of which studied in their microbiological characteristics includes; traditional fermented dairy products *ergo* (sour milk), *ayib* (cottage cheese), *qibe* (traditional butter), *arrera* (defatted buttermilk)

(Almaz Gonfa *et al.*, 2001). Some from traditional fermented plant foods includes *injerra*, *kotcho*, *siljo* (Tetemke Mehari and Mogessie Ashenafi 1995) *awaze*, *datta* (qotchqotcha) (Ahmed Idris *et al.* 2001). Some of the traditional fermented alcoholic and non-alcoholic beverages are *tella* (Samuel Sahle and Berhanu Abegaz Gashe, 1991), *tej* (Vogel and Abeba Gobezie, 1983) *borde* and *shamita* (Tetemke Mehari and Mogessie Ashenafi, 1995).

Ethiopian traditional fermented foods such as *siljo* (Tetemke Mehari and Mogessie Ashenafi, 1995), *datta* and *awaze* (Ahmed Idris *et al.*, 2001) are condiments. Fermented condiments are used as taste enhancers in many traditional dishes. They have constituted a significant proportion of the diet of many people in different parts of the world (Achi, 2005).

Although, a wide variety of fermented foods and beverages are consumed in Ethiopia, the microbiological studies mostly concerned on those foods consumed almost by the majority of people of the country, but microbiological knowhow about those less-known indigenous foods and beverages is limited (Mogessie Ashenafi, 2006). One of these fermented foods condiment is “*azo*” which is prepare from endod’s leaves and sorghum flour. Therefore, this study was undertaken to study the physicochemical and microbial dynamics of less-known indigenous traditional fermented food condiment, *azo*. It is produced by fermentation of endod leaves and sorghum porridge together with other spices and fermentation carried out in traditional container calabash/ gourd locally known as “*banga*” or earthen pot in Tigray regional state particularly Kafta Humera. In this regard, there is no scientific work undertaken on this issue so far. In order to fill this gap, it is necessary to conduct this study in such a way that providing the first insight about the microbiological and some physicochemical profile of *Azo*.

Endod is the Ethiopian vernacular name for the soapberry plant *Phytolacca dodecandra* that is a member of the phytolaccaceae family. The plant is widely distributed in Africa, South America and Asia. This plant has been used as source of detergent and traditional medicine for centuries in Ethiopia (Azene Bekele, 2007). As the result, many researchers conducted on the plant for its medicinal value, chemistry, agronomic aspects and other properties without regard to its food value.

2. OBJECTIVES

2.1. General objective

- ❖ The general objective of this study was to characterize the microbiological and physiochemical profile of *azo*.

2.2. Specific Objectives

- ❖ To determine the physicochemical characteristics such as pH, moisture content and titratable acidity.
- ❖ To examine the nutritional compositions crude protein, fat ash and carbohydrate of fermented *azo*.
- ❖ To examine the microbial load and quality of the *Azo*.
- ❖ To isolate and identify microorganisms which are involved in the fermentation of *Azo*.
- ❖ To determine the microbial succession present in *Azo* during fermentation.

3. LITERATURE REVIEW

3.1. Overview of Fermented Foods

Animal and plant tissues subjected to the action of microorganisms and/or enzymes to give desirable biochemical changes and significant modification of food quality is referred to as fermented food (Sahana and Fauzia, 2003). Fermentation is a process in which microorganism produces chemical changes in organic substrates through the action of enzymes produced by these microorganisms (Li, 2004).

Traditional fermented foods and beverages are product of spontaneous fermentation (Ketema Bacha *et al.*, 2010). This type of process results in microbiological and chemical variability in the products, as it depends on the microflora naturally present in the substrates, on utensils and equipment used or from a previous batch of the fermented products (back slopping) (Steinkraus, 2002). These microorganisms modify the substrates biochemically and organoleptically into edible products that are culturally and socially acceptable to the consumers (Tamang, 2010)

While many studies have investigated concerning traditional fermented foods, still there are about 5000 varieties of major and minor unlisted fermented foods and beverages in the world prepared and consumed by billions of people belonging to different communities and ethnicities. From those, the consumption of some less known and uncommon ethnic; fermented foods is declining due to changes in lifestyle and the shift from cultural foods to commercial foodstuffs and fast foods, and also due to climate change in some places, which affects traditional culinary practices drastically.

Fermented foods are typically unique and vary from region to region due to the variation in

climate, social patterns, consumption practices, microorganisms and most importantly the availability of raw materials (Nout and Mortarjemi, 1997). The main component of fermentation ecosystem are organic matter to be fermented, microbiota, (bacteria, yeasts and molds), the solution in which fermentation take place and fermenting container (Scott and Sullivan, 2008). The microorganisms use the food as a substrate for their propagation (Aderiye and Laleye, 2003; Mosha and Vicent, 2004).

There are four main fermentation processes: alcoholic, acidic and alkali fermentation. Alcoholic fermentation is in which ethanol the main product from the sugar metabolism by yeast. whereas, acidic fermentation characterized by a significant production of acetic acid or lactic acid by the action of acetic acid bacteria and lactic acid bacteria respectively. Vinegar, coffee, wine and cocoa are examples of fermented products where acetic acid fermentation takes place by the conversion of alcohol to acetic acid due to the presence of acetic acid bacteria (De Vuyst *et al.*, 2008; Sengun & Karabiyikli, 2011) lactic acid fermentation is transformation of sugar to lactic acid as main metabolic product. Whereas, alkali fermentation is a proteolytic type and often takes place during the fermentation of fish and seeds rich in proteins. These are popularly used as condiments (Steinkraus, 2002).

Lactic acid bacteria mainly carry out lactic acid fermentation. This type of fermentation performs a number of essential roles, including the preservation and production of wholesome foods. They are generally inexpensive and often little or no heat is required in their preparation. The character and organoleptic properties of these fermented foods depend on microbial activities (Nwosu and Ojmelukwe, 2000). Indeed, the types of microbial flora developed in each of the fermented food depend on water activity, pH, salt concentration, temperature and composition of the food matrix (Blandino *et al.*, 2003). In general, wide spectrums of microorganisms are

involved during fermentation processes but a few types usually determine the quality of the product (Kebede Abegaz, 2007). Given adequate environmental conditions, particular microbial communities determine the quality of a specific food. The origin development and succession of a particular microbial community in any food item are thus governed by ecological factors, which influence the physiological expression of microbial cells including LAB (Park *et al.*, 2010).

The weight of the microorganisms in the food is usually small, but their influence on the nature of the food, especially in terms of flavour, and other organoleptic properties, is profound (Okafor, 2009).

3.2. Function of Fermented Foods

As the production and consumption of traditional fermented food products become increasingly relevant in the face of rapidly increasing population and food insecurity, more research and development to ensure the safety and nutritional quality of these fermented products is warranted. The importance of fermented foods in modern-day life is underlined by the wide spectrum of fermented foods marketed both in developing and industrialized countries, not only for the benefit of preservation and safety, but also for their highly appreciated sensory attributes (Holzapfel, 2002).

Millions of people in the developing world depend on fermentation technology for preservation of their food at costs available to the average consumer (Jakobsen and Lei, 2004; Kalui *et al.*, 2010). Fermentation also markedly improves digestibility, flavor of raw seeds (Yateem *et al.*, 2008), make otherwise inedible foods products edible, enhance the nutritional value, decrease toxicity, preserve food and decrease cooking time and energy requirements (Parkouda *et al.*,

2009).

3.3. Cereal fermentation

Cereals are growing over 73% total world harvested area and contribute over 60% to the world food production. In addition, cereals are almost the only source of nutrition for one-third of the world's population especially for developing and underdeveloped nations of Sub-Saharan Africa and South-east Asia (Sofi *et al.*, 2009). Cereal-based fermented foods are major contributors to energy intake in developing countries. They providing dietary qualities, proteins, energy, minerals and vitamins required for human health. Cereals can be used as fermentable substrates for the growth of probiotic microorganisms (Charalampopoulos *et al.*, 2002). They can be used as sources of non-digestible carbohydrates that besides promoting several beneficial physiological effects can also selectively stimulate the growth of lactobacilli and bifidobacteria present in the colon and act as prebiotics.

There have been many fermented products made from cereals throughout the history of human nutrition. In the course of the fermentation process, the products developed are characterized by quite different properties and uses. A few examples include; leavened baked goods obtained from sourdough and/or yeast leavened dough, African lactic acid bacteria fermented gruels such as *ogi*, alcoholic drinks such as beer, sake, and spirits, acid fermented nonalcoholic drinks such as *boza* (Turkey), *berlinerwei* (Germany), *kwass* (Russia), or *mahewu* (South Africa) (Hammes *et al.*, 2005).

Cereals also used as ingredient for the preparation of fermented condiments such as *miso* (salted paste) (Japan) and vinegar (Europe and America) (Charalampopoulos *et al.*, 2002), *me* (rice acidic sour condiment) ferment by LAB in Vietnam and *Taotjo*, Condiment produce by

fermentation of wheat and/or rice in East India (Tamang, 2010). In Ethiopia, maize can also be used for production of *siljo* (Diriba Geleti *et al.*, 2001) by substituting these familiar ingredients (legume).

Lactic acid fermentation of cereals is a long established processing method and is being used in Asia and Africa for the production of foods in various forms such as beverages, gruels, and porridge (Salovaara, 2000). The good growth of LAB in cereals suggests that the incorporation of a human derived probiotic strain in a cereal substrate under controlled conditions would produce a fermented food with defined and consistent characteristics (Correia *et al.*, 2010).

3.4. Fermented Condiments

Traditional fermented condiments are among the various traditional fermented foods, which are produced by microbial fermentations under highly variable conditions (Odufa, 1985) in different parts of the world. Such food products are not consumed alone, but are added as a condiment to make the food more tasty and enjoyable (Hesseltine, 1983). Apart from the flavoring attributes, they contribute to the protein intake of consumers. Condiments constitute significant proportion of African diets where they serve as flavorsome and culinary components in various dishes (Achi, 2005).

In recent years, the use of fermented food condiments and flavoring agents are becoming popular in the diets of many nations. Apart from the fact that those condiments improve sensory properties of foods and vitamins (Kolapo *et al.*, 2007), some of them contain antioxidants and nutraceuticals that provide health benefits (Omafuvb *et al.*, 2004; Dakwa *et al.*, 2005). In cases where the process of fermentation evolved for the development of taste or aroma, it often result

in enhanced nutritional value by providing dietary fiber, minerals and vitamins (Kolapo *et al.*, 2007).

Fermented condiments also play a vital role in stabilization of the original raw materials, and detoxification of anti-nutrient factors (Rhee *et al.*, 2011). They also have great potential as key protein (Umoh and Oke, 1974), fatty acid, good sources of gross energy; particularly they provide protein for those unable to afford much meat in their diets. It is evident that fermented food condiments are good sources of nutrients and could be used to produce complementary food supplements. Hence, they are basic ingredients for food supplementation and their socioeconomic importance cannot be over emphasized (Achi, 2005), in many countries especially in Africa and India where protein calorie malnutrition is a major problem (Sarkar *et al.*, 1993

In many cases, traditional fermented food condiments are derived from legumes as well as fish, which are rich in protein (Steinkraus, 2002). Therefore, bacteria of the genus *Bacillus* are usually important in non-acidic fermentation of a proteolytic type. They hydrolyze proteins into amino acids and ammonia (Dakwa *et al.*, 2005). In most parts of Africa protein-rich foods oil seeds such as African locust bean, melon seed, mesquite bean and soybean are fermented to give condiments (Omafuvbe *et al.*, 2004). The seeds of African locust bean (*Parkia biglobosa*) are fermented to produce “dawadawa” or “iru” in Ghana and Nigeria. The seeds of melon (*Citrullus vulgaris*) are fermented to produce *ogiri* in Nigeria and Sierra Leone (Kpikpi *et al.*, 2009). Analogously, in our country a popular fermented food condiment known as ‘*siljo*’ is made from fermented safflower (*Carthmu stinctorius*) and horse bean (*Vicia faba*) (Tetemke Mehari and Mogessie Ashenafi, 1995).

Although; in most parts Africa including Ethiopia, protein rich food ingredients are often fermented to make condiments, low protein ingredients also fermented into condiment in Ethiopia (Mogessie Shenafi, 2002). These includes 'awaze' and 'datta' which are made from red sweet pepper (*Capsicum annum*) and small chili pepper (*C. frutescence*) at its green stage, respectively as main ingredients (Ahmed Indris *et al.*, 2001).

Previous studies reported that the fermentation processes of awaze datta primarily carried out by lactic acid bacteria however, other bacterial species such as aerobic mesophilic, coliforms, enterobacteriaceae and yeasts were also isolated. Ingredients for awaze preparation had a microbial load of 10^6 cfu/g and the flora was dominated by *Bacillus* species. The count of aerobic mesophilic bacteria decreased during the fermentation period. Lactic acid bacteria reached the maximum count of 10^9 CFU/g at day 4 and the count remained $>10^8$ CFU/g end of fermentation. The heterofermentative lactic acid bacteria dominated until day 3 and the homolactics took over thereafter. Yeasts also reached counts of 10^6 cfu/g at the end of fermentation on day 12. A steady decline in pH was observed in the course and this was accompanied by increase in titratable acidity. In datta fermentation, the count of aerobic mesophilic bacteria remained unchanged during the fermentation. Lactic acid bacteria initiated the fermentation at a level of 10^4 cfu/g and reached 10^9 cfu/g at end of fermentation on day 7. Unlike awaze fermentation, the homofermentative lactic acid bacteria initiated and dominated datta fermentation for the first two days. The heterolactics dominated thereafter (Ahmed Idris *et al.*, 2001).

In case of siljo fermentation, aerobic mesophilic bacteria and lactic acid bacteria were present at levels as high as 10^{10} CFU/ml after 36 h of fermentation, but Enterobacteriaceae were not detected. *Lactobacillus* was dominating LAB during the fermentation process. The moisture

content of siljo was about 86%. Furthermore, a slight improvement in crude protein and crude fat was observed during the fermentation (Tetemke Mehari and Mogessie Ashenafi, 1995)

3.5. Lactic Acid Bacteria (LAB)

Lactic acid bacteria (LAB) were first described as milk-souring organisms, due to the sour milk that arose from their production of lactic acid (Zotta *et al.*, 2009). They are the most important microorganism in the food industry. To date, food industries used LAB in fermentation processing for prolonging the shelf life of perishable raw materials, influence the flavor, texture, nutritive properties, health attributes and commercial value of traditionally and industrially fermented foods (Patrick, 2012).

LAB are considered to have several beneficial physiological effects, such as enhancing of immune potency (Kullisaar *et al.*, 2002) and prevention of cancer and lower serum cholesterol levels (Kaur *et al.*, 2002). LAB also have beneficial influence on nutritional and sensory characteristic as well as on the standardization of end product (Gawad *et al.*, 2010). Furthermore, they play a vital role in antagonistic activities of human pathogens directly through production of secondary metabolites such as organic acids, bacteriocin and bacteriocin-liked inhibitory substances (Girum Tadasse *et al.*, 2005), which is good solution to address the current serious problem of drug resistant (Cueto *et al.*, 2007). Thus, recently receive higher attention of many researchers to achieve nutritional and health problem in the world (Temmerman *et al.*, 2002).

Lactic acid bacteria are the most widely used bacteria as starter cultures for the industrial processing of fermented dairy, meat, vegetable and cereal products. However, many kinds of foods are still fermented naturally, without the use of starter cultures by autochthonous lactic acid bacteria, which form characteristics properties of products (Suskovi *et al.*, 2010).

3.5.1. Classification of LAB to genus level

The general description of the bacteria within this group is gram-positive, non-sporulating, non-respiring cocci or rods, which do, through fermentation of carbohydrates, produce lactic acid as their major product. LAB are chemotrophic and find the energy required for their entire metabolism from the oxidation of chemical compounds (Khalid, 2011).

Lactic acid bacteria consist of a number of bacterial genera within the phylum Firmicutes. The genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Milissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, *Weissella* (Girum Tadesse *et al.*, 2005) *Aerococcus*, and *Paralactobacillus* are recognized as lactic acid bacteria (Patrick, 2012).

After the work by Orla-Jensen, the view emerged that the core of lactic acid bacteria comprised four genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* (*Lactococcus*, *Enterococcus* and *Streptococcus*) (Sangoyomi *et al.*, 2010). This classification of LAB into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures and configuration of the lactic acid produced, ability to grow at high salt concentrations and acid or alkaline tolerance (Hutkins, 2006).

3.5.1.1. Lactobacilli

The genus *Lactobacillus* is the largest genus of LAB group currently consists of over 152 validly described species. They are usually straight, although they can form spiral or coccobacillary forms under certain conditions. They are often found in pairs or chains of varying length. They are known resisting acidic pH 3.5 to 4, aciduric or acidophilic better than other genus of LAB (Goyal *et al.*, 2012). Based on their carbohydrate fermentation *Lactobacillus* subdivided into three groups:

i. Obligate homofermentative Lactobacillus

The obligate homofermenters include species such as *Lb. acidophilus*, *Lb. delbriickii* and *Lb. helveticus*. They ferment hexoses almost exclusively to lactate but are unable to ferment pentoses. (Ray, 2004).

ii. Facultative heterofermentative Lactobacillus

The facultative heterofermenters ferment hexoses via the EMP pathway to lactate but have an inducible phosphoketolase that allows them to ferment pentoses to lactate and acetate. They include some species important in food fermentation such as *Lb. plantarum*, *Lb. casei*, and *Lb. sake* (Ray, 2004).

iii. Obligate heterofermentative

They fermenting hexoses to lactic acid, acetic acid, ethanol, CO₂, and ferment pentoses to lactic acid and acetic acid (Amin *et al.*, 2009). This group can be distinguishing from other heterofermentative LAB by their ability to hydrolyze arginine. Heterofermentative Lactobacillus have been recently reclassified in the new genus *Carnobacterium* (Adams and Moss, 2000)

3.5.1.2. Pediococcus

The genus *Pediococcus* encompasses homofermentative cocci which produce lactic acid as major end product of glucose fermentation. The cells are uniformly spherical and never ovoid or elongated and they differ from all other lactic acid bacteria by alternate division in two perpendicular directions, resulting in tetrad formation (Franz *et al.*, 2007) but sometimes they can occur in pair and chain. They can grow at 45°C but best at 32°C. *Pediococcus* have been found growing, during the fermentation of brined vegetables (Piva and Headon, 1994). The once

Ped. halophilus now in the genus *Tetragenococcus*. It can grow in 18% NaCl (Adams and Moss, 2000)

3.5.1.3. Streptococcus

Members of the diverse genus *Streptococcus* were reclassified into *Lactococcus*, *Enterococcus*, *Vagococcus*, and *Streptococcus* based on biochemical characteristics, as well as molecular features. Many species of this genus are parasite to man, but *Streptococcus thermophiles* is a thermophilic lactic acid bacterium (LAB) widely used as starter in the manufacture of dairy products (Delorme, 2008). This species closely related to *Lc. lactis*, but it is even more closely related to other streptococcal species including several pathogens (Mitchell, 2003). The cells are spherical or ovoid. The organism does not grow at 15⁰C, but most strains are able to grow between 40⁰C and 50⁰C. Its optimal growth rate occurs at the bottom of the thermophile spectrum, around 45⁰C (http://web.mst.edu/~microbio/BIO221_2010/S_thermophilus.html).

This property is useful for much industrial dairy fermentation requiring the process of milk at higher temperatures. *Streptococcus* species are well survived at reduced pH levels next to *Lactobacillus* (FDA, 2011).

3.5.1.4. Enterococcus

Enterococcus is another genus of LAB formally categorized under streptococci, which represents 6% of the fecal microbiota and 23% of the microbiota in the cecum at the junction of the ileum and colon (Pavan, and Pot, 2003). Nevertheless, they also found in fermented foods and are frequently isolated from starter cultures and cheese producers. They are typical homofermentative cocci, which can grow at 10⁰C and at 45⁰C, in the presence of 6.5% NaCl concentration and at pH 9.6. Hence, the capacity to grow in broths at high pH, high salt concentrations, and a wide temperature range (10⁰C to 45⁰C) differentiated them from *Streptococcus* and *Lactococcus*

(Facklam *et al.*, 1999).

3.5.1.5. Lactococcus

Lactococcus is a genus of lactic acid bacteria that were formerly included in the genus *Streptococcus*. They are morphologically spherical or ovoid cells that occur singly, in pairs, or as chains. They grow at 10°C but not at 45°C. Hence, this character is used for distinguishing them from *Streptococcus* and *Enterococcus* (Adams and Moss, 2000).

3.5.1.6. Leuconostoc

Leuconostoc are mesophilic, heterofermentative, diplococci in oval chain, which are known by their ability to produce gas from glucose. They are typically found in association with the heterofermentative *Lactobacillus*; they are distinguished by their incapacity to hydrolyze arginine (Adams and Moss, 2000). The genus reduced in number of species. The former *Leu. oenos* has been transferred to genus, *Oenococcus*, *O. oeni*, and the former *Leu. pmaramesenteroides* has been transferred to the new genus *Weissella* (Ray, 2004).

Table 3.1: LAB classification to genus level (Nikita and Hemangi, 2012).

Gram positive, catalase negative and oxidase negative						
Gas production from glucose						
Negative				Positive		
Cocci				Rod	Cocci and arginine hydrolyze (-)	Rod and arginine hydrolyze (+)
Tetrad						
Positive		Negative				
Growth at						
		15 ⁰ C	45 ⁰ C	6.5 %	pH 9.6	
Pediococcus		+	-	+	+	Lactococcus
		+	+	+	+	Enterococcus
		-	+	-	-	Streptococcus
				Lactobacillus	Leuconostoc	Lactobacillus

Whereas +=positive, -=negative

3.6. Fermented Foods in Ethiopia

Based on its rich cultural diversity, a wide variety of fermented foods and beverages are consumed in Ethiopia from different raw materials such as cereals, ensete (false banana), honey, milk, etc. (Mogessie Ashenafi, 2006). The products, if properly exploited, could be of significant economic importance for the country. These foods are relatively cheap to prepare and are therefore important alternatives for low-income consumers who cannot afford imported or industrially processed foods and beverages. In addition, a more effective and rational production could give increased income for those who are involved in the production (Kebede Abegaze *et al.*, 2002).

Food preparation is predominantly a household phenomenon in Ethiopia. The creation of the right environment for the microorganisms to result a desirable product is based on women's indigenous knowledge, which has improved itself through generations (Mogessie Ashenafi, 2002). As the result, the outcome and quality of spontaneous food fermentation may not be predictable (Askal Desiye and Kebede Abegaz 2013).

3.7. Endod (*P. dodecandra*)

The plant of Phytolaccaceae family encloses 35 species with a close relativity and similar characteristics. These different species are used in same applications (Ravikiran *et al.*, 2012).Of these, Endod (*P. dodecandra*) widely grow in Sub-Saharan Africa, including Ethiopia which is found at altitudes that range between 1600 and 3000 meters above sea level (m.a.s.l.) (Legesse Wolde-Yohannes, 1983). Since the discovery of the molluscicidal properties of endod in 1964, more than 40 scientific articles have been published on different aspects of the plant (Aklilu Lemma *et al.*, 1991).

Parts of the endod plant have been used for different socio-economic aspect in Ethiopia (Azene Bekele, 2007). The berries are used as detergent and soap for washing. The roots and leaves, despite their toxicity have been reported to have medicinal value for various ailments, including termination of unwanted pregnancies. It has molluscicidal and spermicidal properties and is used to control schistosomiasis, Zebra mussels and mosquito larvae (Endashaw Bekele, 2007). In addition the protein extract from Pokeweed (*Phytolacca americana*) have antiviral activities against several animals and plants viruses ([http: www.eura.org/emea.html](http://www.eura.org/emea.html)).

The fact that, several toxins have been identified in different *phytolacca* species, usually concentrated in roots, berries and seeds although; all other parts of the plant contain smaller amounts of the toxic principles. These poisons include an alkaloid (phytolaccine), a resin (phytolaccatoxin), and a saponin (phytolaccigenin). Despite the presence of those toxins, the plant is occasionally used as vegetable in several regions of the world. Therefore, poisoning incidences were reported rather frequently but symptoms were normally mild and case of death rare.

In India, *Phytolacca acinosa* leaves required some special treatment in order to reduce the bitterness, through boiling and washing several times before the final preparation of vegetable. Wild vegetables were nutritious as they contained high protein and other nutrients, which helped in the food security of rural people (Sundriyal and Sundriyal, 2004). Similarly, In North America) freshly cut young leaves and shoots of *P. americana* may be cooked and eaten like spinach. They should be boiled twice, and the first water being discarded. The leaves are also processed in a similar manner for use as tea. In addition, the young shoots are also eaten as a substitute for asparagus. The red juice of the berries is extracted and then cooked, and then used in the making of pies. It is also added to other juices that are used for making jelly. Wine

grower also took the red fruits for coloring (and adulterating) <http://www.naturalremedies.org/po-keweed/2014/10/27>).

In Ethiopia, it has been indicated that extracts of this plant are sometimes added to local drinks such as *tej*. Such practices might have undesirable side effects on the people who consume those fermented products however; no scientific studies have conducted concerning the negative side effects. Another local sauce, *Wo'ett*, is also prepared from endod in some parts of Ethiopia (Amare Getahun, 1976).

3.8. Preparation of Azo

Azo is a traditional fermented semi-solid condiment, which is consumed during Easter fasting season in western Tigray region, particularly in Kafta and Welkait as well as in Gonder. Of course, the way of processing and ingredients added for the preparation of this condiment differ from one community to other. In Kafta, cereal flours and, fresh leaves of endod are primary ingredients used for *Azo* preparation along with different variety spices namely rue, garlic, ginger, korerima, black cumin, black mustards and as minor ingredients for flavour enhancement.

During preparation of *Azo*, the leaves of *P. dodecandra* collected from the field are washed thoroughly to remove dusts and waste materials. After that, washed leaves are cooked for 30 minutes and left to cool. This cooled preparation is washed with tap water until the soupy material is completely removed. Then, it is wet milled with traditional stone mill. At the same time, the cereal flour is cooked to form porridge and allowed to cool for some time. The porridge, wet-milled slurry endod leaves, and the spices are mixed and transferred to smoked traditional utensil made from *Cucurbita teta* (calabash/gourd) locally called *banga* or earthen pot

and covered tightly. Finally, this mixture is allowed to ferment at ambient temperature for 5-7 days, it becomes ready for consumption as Azo. This fermented condiment has a sore test and desirable aroma, which is influenced by the types and amount of spies added and the level of exposure of storage to smoke. Smoking provides the food to have a good taste, which provide flavour like cheese and this taste is accepted by the local people as good quality. Smoking is usually carried out by *Dichorostachys cinerea* locally called “Gonok”.

3. MATERIALS AND METHODS

The samples for this study were collected from Kafta Humera, one among the districts of Tigray regional state, which is located in the northwestern Ethiopia (MAP). Studies on the physicochemical and microbial profile of *Azo* were conducted at the Food Microbiology and Food Safety Research Laboratory at the Ethiopian Public Health Institute (EPHI), Addis Ababa.

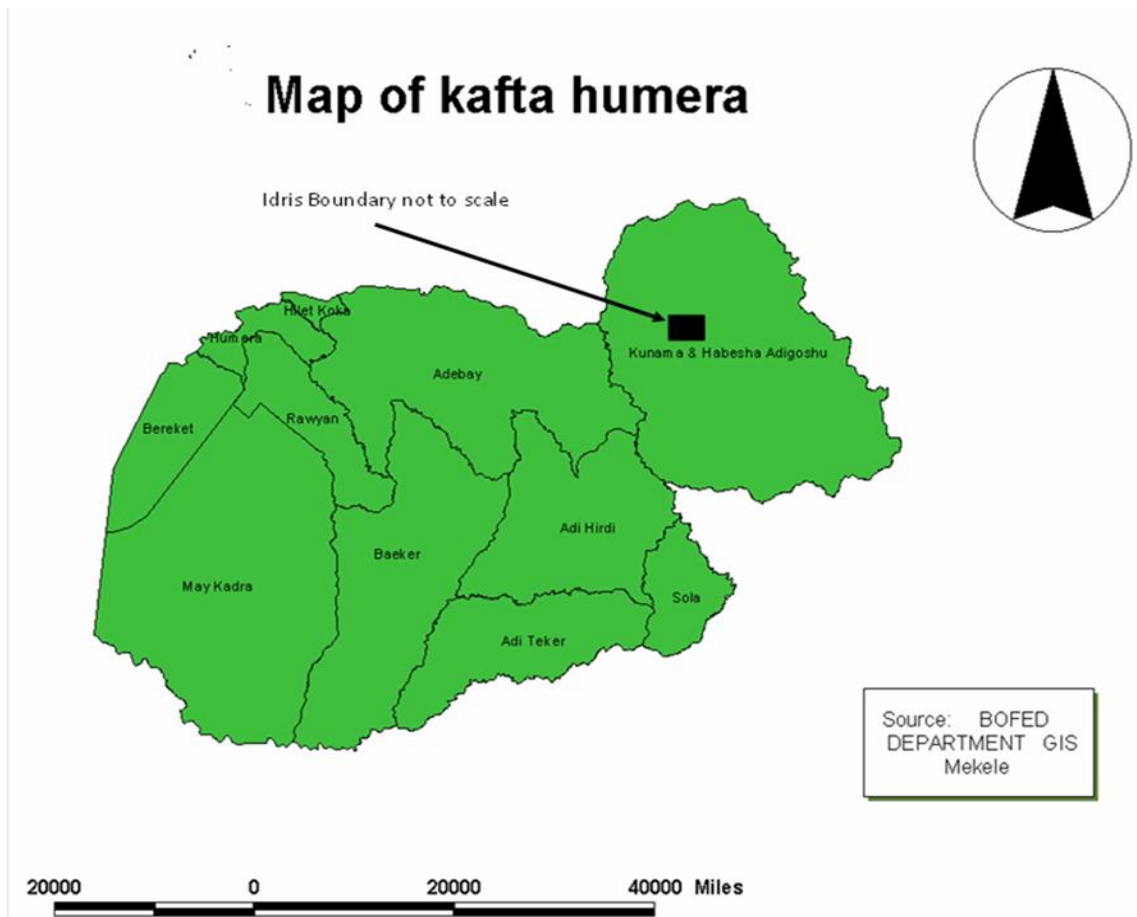


Figure 4.1: Map of Kafta-Humera district (Haile Fesseha, 2007)

4.1. Sample Collection and Preparation

4.1.1. Sample collection

Four samples were taken from household products collected from Kafta (AZOII, AZOIII, AZOIV, and AZOV) using sterilized screw bottles in the icebox (December 2013). Samples were kept in a refrigerator at 4⁰C until processing. All of them were taken from a 6th day fermentation time. The comparative study was undertaken on five samples of which one sample was taken from a laboratory prepared Azo (AZOI), and the other.

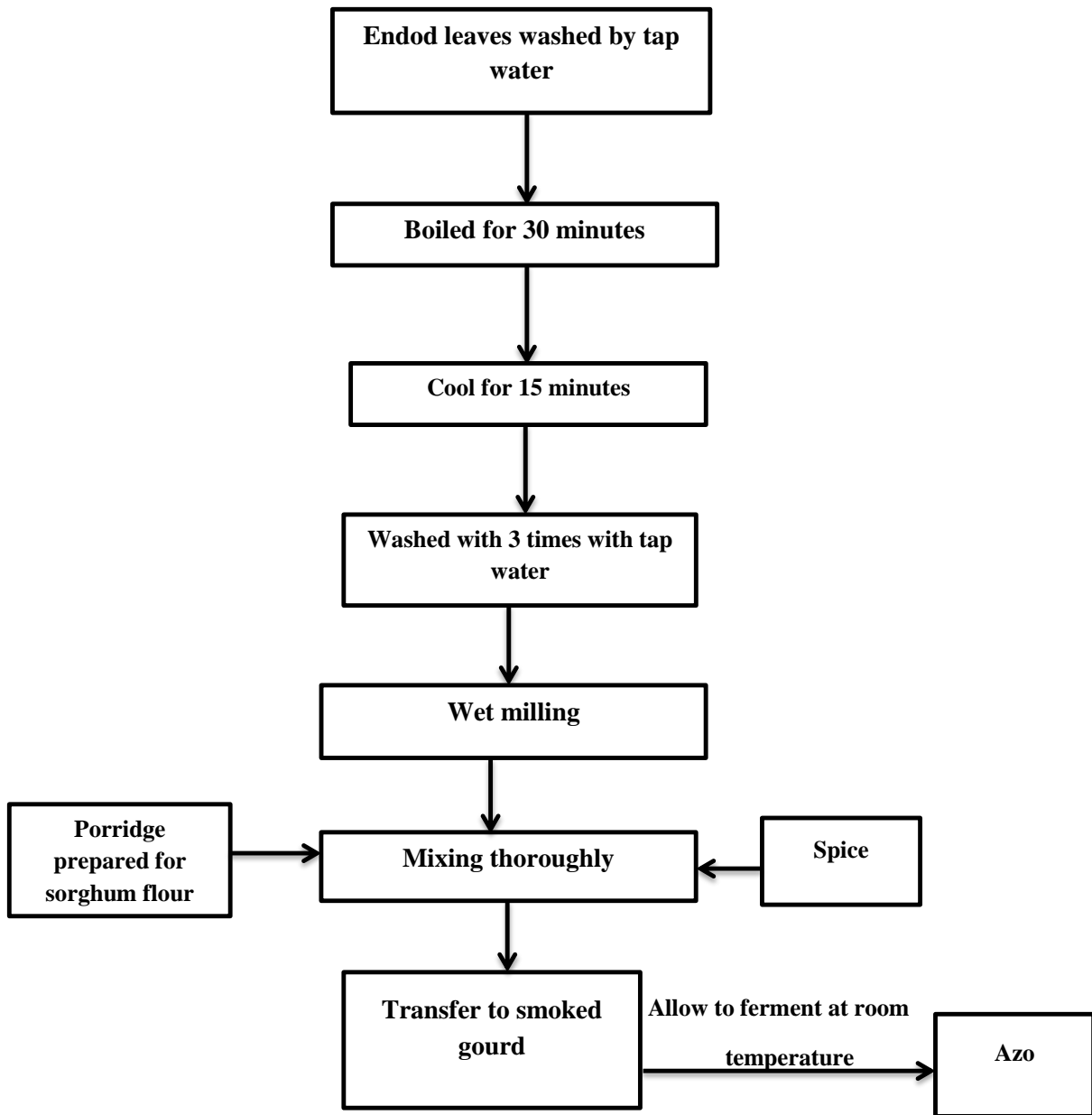
4.1.2. Sample preparation in laboratory

Azo (AZO I) was fermented using the same procedure as that of the traditional fermentation. The fermentation undertaken for 30 days taking samples initially and every 24hrs for the first 7 days and two samples were taken at day 15 and day 30. Four hundred and fifty (450) g *P. dodecandra*, 450g sorghum, 75g mustard and 43g spices was used to *azo* preparation in the laboratory described in figure 4.1. These spices were prepared from different ingredients listed below in table 4.1.

Table 4.1: List of spices for used Azo preparation in the laboratory

Vernacular name		Common and scientific names	
Tigrigna	Amharic	Common names	scientific names
Tsadshengurti	Netchshinkurt	Garlic	<i>Allium sativum</i>
Chn-adam	Ten-adam,	Rue	<i>Ruta graveolence</i>
Zingebile	Zingebile	Ginger	<i>Zingiber officinale</i>
Korerma	Korerma	Korarima	<i>Aframomum korerima</i>
Seseg	Zekakibe	Basil	<i>Ocimum basilium</i>
Azmut	Netch-azmut	Bishop'sweed	<i>Trachyspermum ammi</i>
Aweseda	Tikur-azmut	Black cumin	<i>Nigella sativa</i>
Senafitch	Senafitch	Black Mustard	<i>Brassica nigra</i>

Fig4.2: Schematic representation of steps involved for the preparation of Azo in laboratory



4.2. Physicochemical analysis of Azo

4.2.1. PH

Five g of Azo samples were mixed and homogenized with 45 ml distilled water and pH were measured using a digital pH-meter (AOAC, 2005).

4.2.2. Titratable acidity

Titrate acidity expressed as percent of lactic acid, was determined according to the methods described in (AOAC, 2005) by titrating Azo samples using 0.1N NaOH solution and phenolphthalein as indicator. It was calculated as:

$$\% \text{Lactic acid} = \frac{\text{ml of NaOH} \times \text{M of NaOH} \times \text{molecular mass of LA}}{\text{Volume of sample} \times 10}$$

Where molecular mass of LA=90.08mg

4.2.3. Determination of Moisture Content

Moisture content of the samples was analyzed by oven drying method (AOAC, 2005). Five grams of sample were transferred to clear and dry Petri dish with defined weight. Then the dish was dried in an oven at 105°C for 2h and placed in desiccators to cool the samples at room temperature and reweighed again until a constant weight were obtained. The percentage moisture content was calculated:

$$\text{Moisture content (\%)} = \frac{\text{loss in weight due to drying} \times 100}{\text{Weight of wet samples}}$$

4.2.4. Ash content

The ash content was estimated by incineration with the furnace at 550⁰C (AOAC, 2005).

4.2.5. Crude fat content

The Soxhlet fat extraction methods was used to determine the fat content of Azo samples as described by AOAC (2005) method. Three grams of sample was fed into a Soxhlet apparatus fitted with a 1-L round-bottomed flask and a condenser. The extraction was carried out for 8 h

using n-hexane (boiling point 68-70⁰C). The solvent was removed by heating at 70⁰C in a hot dry oven. The recovered oils was then weighed and expressed as percent fat (Eyassu Seifu, 2013).

$$\mathbf{WF= WA- WB}$$

$$\mathbf{\% \text{ fat g/100}=\frac{WF}{WB} (100-\text{moisture } (\%))}$$

WB

Whereas: WA=weight of flask after extraction

WB= weight of flask before extraction

WD = weight of dry sample after moisture determination

WF=weight of fat

4.2.6. Crude protein content

The total nitrogen content of 1 g sample was determined as described by the Kjeldahl method using Kjeldat apparatus (Kjeldatherm KB/KB). The crude proteins were calculated by multiplying nitrogen content by a factor of 6.25 (AOAC, 2005).

4.2.7. Carbohydrate content

The carbohydrate content was determined by differences: 100 - (moisture+% protein + % fat + % ash) (AOAC, 2005).

4.3. Enumeration of microbial groups

Samplings were performed at 0 h and then every 24h for 7 days as well 15 and 30 days to examine the microbial safety. 10g *Azo* Samples were taken aseptically and homogenized in sterile 90ml peptone solution 0.1% (w/v) containing 10%(w/v) NaCl (85 ml) for 1 minute. Then

plate counts were carried out using the following media, temperature and incubation periods to enumerate different microbial group.

4.3.1. Total viable bacteria count

To determine the total bacterial count 0.1 ml of serially diluted 0.1% (w/v) sample was inoculated Standard plate count agar (PCA) (Oxoid) and incubated at 30-32°C for 48h. Then colony count was made using colony counter (Shreve and Arils,2003).

4.3.2. Lactic Acid Bacteria Count

From appropriate dilutions, 0.1 ml aliquots were spread plated in triplicates on pre-dried surfaces of MRS (de-Mann, Rogosa and Sharp) agar (Oxoid) plates. The plates were incubated at 30-32°C for 48hrs (Soda *et al.*, 2003).

4.3.3. Total Coliform Count

To enumerate total coliforms 0.1ml of serially diluted sample were used and inoculate Trypticase soy agar (TSA) (Oxoid) after 30 minute overlay with violate read bail agar (VRBA) medium (BFCO). The plates were incubated at 37°C for 24 h. Then the count was under taken by using colony counter (Todd *et al.*, 1997).

4.3.4. Fecal Coliforms Count

Fecal coliforms counts were determined by TSA-VRBA overlay method and the plate incubated at 44 °C for 24 h (Todd *et al.*, 1997)

4.3.5. Yeasts and Moulds Count

Yeast and mould were counted by inoculating 0.1ml sample on PDA (BFCO) medium after having incubated them at 25°C for 5 days (Barrow and Feltham 1993).

4.3.6. Detection of Salmonella

The stage of the pre-enrichment of *Salmonella* medium was done by mixing 25 g of sample with 225 ml of buffer peptone water in a sterile flask and incubated at 37°C for 24 hours. Then the selective enrichment media was done by preparing 1ml of the pre-enrichment culture mixing with 10 ml of selective selenite broth and incubating at 37°C for 24 hours. A loopful culture from selective enrichment broth was transferred to selective agar media (xylems lysine deoxycholate (XLD) agar and incubated at 37⁰C (Ahmed Indris *et al.*, 2001)

4.4. Isolation and Purification LAB

The MRS agar medium and broth were used as selective media for isolation and purification LAB. Accordingly, the diluted samples were pour plated on the MRS agar plates and incubated in anaerobic jar, at 30-32⁰C for 72h. The typical colonies on MRS agar were sub-cultured twice overnight in MRS broth and subsequently transferred s to MRS agar for purity (Kebede Abegaze, 2007). The isolates were classified to the genus level based on morphological, biochemical and physiological characteristics (Nikita and Hemangi, 2012).

4.4.1. Preliminary Characterization

4.4.1.1. Cell Shape and Cell Arrangement

Overnight pure broth cultures were smeared on clean microscopy slide and observed under phase contrast microscopy for their cell shape and arrangement (Kebede Abegaz, 2007).

4.4.1.2. Motility test

Motility was studied using Sulphur indole motility solution on MRS medium according to Okoro *et al.*, (2011).

4.4.1.3. Gram reaction

Each isolate was smeared on grease free slide, air-dried and heat fixed by passing each slide over the blue flame of a burning Bunsen burner repeatedly. Each slide was flooded with a crystal violet solution, dried for a minute, washed with distilled water and treated with iodine for a minute. The slides were decolorized by ethanol, washed with distilled water and counter stained using safranin for 30sec. They were washed again, air-dried, and observed under oil immersion objective lens (x100) of the light microscope (Onyeagba, 2004).

4.4.1.4. Catalase Test

Catalase test was performed by transferring bacterial culture on slide containing a drop of 3% solution of hydrogen peroxide (H₂O₂). The formation of bubble was a positive test for catalase within few minutes (Kimaryo *et al.*, 2000).

4.4.1.5. Oxidase Test

The oxidase test was undertaken on filter paper (Whatman) placed into a petridish and soaked with 0.5 ml of 1% tetramethyl-p-phenylene diamine dihydrochloride. The fresh cultures were streaked onto filter paper. The development of a deep blue color at the inoculation site within 5-10 seconds indicated a positive result of oxidase test (Kimaryo *et al.*, 2000).

4.4.2. Biochemical and Physiological Characterization

4.4.2.1. Gas Production from Glucose

In order to determine the homofermentative and heterofermentative characteristics of isolates CO₂ production from glucose was tested (Kamruzzaman *et al.*, 2013). MRS broth and inverted Durham tubes were prepared and inoculated with loopful of overnight fresh culture and

incubated at 30-32⁰C for 48hr. The production of bubbles in the Durham tubes was indicative of gas production.

4.4.2.2. Growth at Different NaCl Concentration

The ability of isolate to grow at different NaCl concentration was tested by inoculation loopful of overnight culture on taste tube containing MRS broth adjusted to 2, 4, 6.5, 8 and 10 % NaCl against control (without salt). Then, they were incubated at 30-32⁰C for 48 h to observe growth based on turbidity of the broth (Chowdhury *et al.*, 2012).

4.4.2.3. Growth at Different pH

The growth of the isolates at different pH was determined by inoculating loopful of overnight culture into MRS broth adjusted to pH 3.9 and 9.6. They were incubated at an anaerobic condition at 30-32⁰C for 48hr. Growth was determined by observing their turbidity on the broth medium (Chowdhury, et al. 2012).

4.4.2.4. Growth at Different Temperatures

A loopful of overnight cultures were inoculated in MRS broth and incubated at 10 °C, 15°C, 37°C and 45°C for 24 h in order to determine their growth according to Tambekar and Bhutada (2010).

4.5. Data Analysis

Means and standard deviations were calculated for microbial count and physicochemical using IBM SPSS, 2012 version 21 software. ANOVA and Kruskal Wallis test was conducted for microbial and physicochemical analysis respectively. The comparisons for each mean was performed by using Duncan's multiple range tests ($\alpha= 0.05$).

5. RESULTS AND DISCUSSION

5.1. Approximate Composition of Azo fermented in the laboratory

The approximate physico-chemical analysis of Azo prepared in the laboratory is presented in Table 5.1. The result indicated pH, titratable acidity, moisture, protein, ash, fat and carbohydrate content varied at different fermentation periods. Accordingly, there was significant difference ($P < 0.05$) in pH value as fermentation progressed. The initial pH (day 0) of fermenting sample was 6.58. Then after the pH value were measured 5.80, 4.82, 3.97, 3.91, 3.87 and 3.81 at day 1, 2, 3, 4, 5 and 6 respectively. the pH value of fermenting Azo started dropping from day 1 and progressively dropped until day 6 (pH 3.81) and remained constant on the 7th day as well as at day 15 and 30 the variation of pH were insignificant (Table 5.1).

The pH reduction in the present study was slightly similar to siljo studied by Tetemke Mehari and Mogessie Ashenafi, (1995) with pH reduction from 6 to 3.9 within 8 days of fermentation and in *awaze* studied by Ahmed Idris *et al.*, (2001) reduced from pH 5.3 to 3.78 from day 0 to day 13 of fermentation time respectively.

Likewise, the titratable acidity increased more than five times from 0.07 to 0.36 of lactic acid up to 7 days; then after it decreased by 21% after 15 and 35% after 30 days of fermentation (Table 5.1). In general, The TA value was similar to the TA value (0.13 to 0.38 %) of the fermented condiment “Awaze” and higher than the TA (0.04% to 0.14%) recorded from condiment “data” (Ahmed Idris *et al.*, 2001), and lower than the ones reported from the traditional fermented siljo (0.36 and 0.75%) (Tetemke Mehari and Mogessie Ashenafi, 1995)

Table5.1: approximate physicochemical analysis of laboratory prepared Azo sample (%)

DF	Fat (%)	Protein (%)	Ash (%)	Moisture (%)	Carbohydrate (%)	pH	TA (%)
0	4.19±0.20 ^b	3.28±0.04 ^g	2.86±0.02 ^a	72.23±0.05 ^j	17.45±0.31 ^a	6.58±0.02 ^a	0.06±0.01 ^g
1	4.60±0.04 ^a	3.47±0.08 ^f	2.73±0.03 ^b	74.14±0.13 ⁱ	15.06±0.28 ^b	5.80±0.01 ^b	0.09±0.01 ^f
2	4.69±0.08 ^a	3.61±0.03 ^{ef}	2.67±0.02 ^b	76.67±0.03 ^h	12.37±0.16 ^c	4.82±0.02 ^c	0.15±0.01 ^e
3	4.74±0.02 ^a	3.68±0.07 ^{de}	2.44±0.03 ^c	78.83±0.07 ^g	10.32±0.19 ^d	3.97±0.01 ^d	0.24±0.01 ^d
4	4.74±0.06 ^a	3.97±0.05 ^c	2.27±0.03 ^d	79.58±0.19 ^f	9.45±0.33 ^e	3.91±0.01 ^e	0.29±0.01 ^c
5	4.59±0.15 ^a	4.56±0.02 ^b	2.11±0.03 ^e	80.06±0.08 ^e	8.69±0.27 ^f	3.87±0.01 ^f	0.34±0.01 ^b
6	3.75±0.07 ^b	4.87±0.02 ^a	2.11±0.05 ^e	80.60±0.06 ^d	8.68±0.20 ^f	3.81±0.02 ^g	0.36±0.01 ^a
7	3.55±0.11 ^c	4.85±0.15 ^a	1.91±0.05 ^f	81.46±0.02 ^c	8.25±0.33 ^f	3.81±0.02 ^g	0.37±0.01 ^a
15	3.25±0.03 ^d	3.84±0.10 ^{cd}	1.72±0.06 ^g	83.71±0.02 ^b	7.49±0.21 ^g	3.69±0.00 ^h	0.29±0.01 ^c
30	2.59±0.04 ^f	3.47±0.04 ^f	1.69±0.07 ^g	84.87±0.01 ^a	7.39±0.16 ^g	3.68±0.01 ^h	0.24±0.01 ^d

- DF= days of fermentation
- TA= titratable acidity
- SD= standard deviation
- Values with the same superscript letter in a column are not significantly different ($P \leq 0.05$).
- Values with different superscripts in the columns differ significantly ($p > 0.05$).
- Values are means of triplicate determinations \pm SD

The data also showed variation in protein, carbohydrate, ash, and moisture content in the lab fermented Azo at different fermentation time (Table 5.1). Accordingly, the fat, ash, and carbohydrate contents were significantly reduced at day 6 and 7 days of fermentation. On the contrary, the protein content increased by 48% after 7 days of fermentation time.

The increase in protein content of Azo is in agreement with the increase in protein content (27%) from day 1 to days 3 of millet (*Pennisetum americanum*) fermentation (Onweluzo and Nwabugwu, 2009). Similarly, Igbabul *et al.*, (2014) reported a 20% increase in protein content of coconut

(*Colocasia esculenta*) fermentation from day 0 to days 3. This is contrary to the report on siljo where fermentation did not change the protein content (27%) from day 0 to the 4th days, (Tetemke Mehari and Mogessie Ashenafi 1995). Other studies on Awaze (Ahmed Ideris *et al.*, 2001) and in *Kawal*, traditional fermented cassava (*Cassia obtusifolia*) leaves from Chad showed a decrease in protein content by 36% during the fermentation process (Mbaiguinam *et al.*, 2005).

At the first three days of fermentation the fat content were increased although, the variation were insignificant ($P > 0.005$). Initially, the fat content was recorded 4.19% then increased for the first three days of fermentation by 13% to reach 4.74 % and remained so for twenty four hours and then began to decrease significantly ($P < 0.05$) afterwards by 45% to reach 2.59 % on the 30th day of fermentation (Table 5.1). The result is similar to the increase in fat content by 19 % (21-25%) of siljo fermentation for the first 4 days of fermentation reported by Tetemke Mehari and Mogessie Ashenafi (1995). The increase in fat content (0.48 to 0.67 %.) was also recorded from fermented coconut during the first few days of fermentation (Igbabul *et al.*, 2014). On the contrary, a 12% and 38% decrease in fat content was recorded in fermented cassava (staple food, “*Gari*”) Irtwange and Achimba (2009) and fermented pigeon pea respectively (Adebowale and Maliki, 2011).

The ash content also significantly affected by the fermentation time ($P < 0.05$) as presented in Table 5.1. It was observed that the ash content reduced from 2.86 ± 0.02 to 1.69 ± 0.07 as fermentation time extended from 0 to 30 day. Atti (2000) and Igbabul *et al.* (2014) have reported similar observations of ash content decrease from 2.70 to 2.68% and from 4.82 to 1.91% in fermented millet and cocoyam flour respectively. Mihiret Kassa (2009) also observed that ash content significantly decreased after sorghum fermentation. On the contrary, ash content of

Nigerian traditional fermented staple food made from Sesame (*Sesamum indicum*) seeds increased from 4.41 to 5.83% from 0 to 4th days of fermentation (Olagunju and Ifesan, 2013). Whereas, Tetemke Mehari and Mogessie Ashenafi (1995) observed that ash content is not affected during siljo fermentation, 7 % ash were observed from 0 to 4th day of fermentation.

The moisture content significantly increased from the start up to the end of the experiment by 17% (Table 5.1). The data showed a steady decrease in pH, TA, ash and carbohydrate contents and an increase in moisture content and protein although, the protein content decreased after 7 days of fermentation. Similar observations have been reported in pigeon pea and gari, fermented cassava roots by Adebowale and Maliki (2011), Irtwange, and Achimba (2009) fermentation increase in moisture content of by 22% and 15 % respectively from day one to day 5. Contrary to this study, moisture content was reported to decrease by 50% during coconut fermentation (Igbabul *et al.*, 2011).

Fermentation significantly ($P < 0.05$) decreased the amounts of carbohydrate of *azo* samples, the values ranged from 17.44 to 7.35. Here fermentation reduces carbohydrate content of the product by 58 % from 0 to 30 days (Table 5.1). Taye Bezuneh (1984) reported that fermentation reduced carbohydrate content of enset from 50% to 41% during kotcho fermentation. Similarly, several researchers reported that carbohydrate content decrease with fermentation period increased (Assouhoun *et al.*, 2013; Kakou *et al.*, 2010 and Onweluzo and Nwabugwu, 2009). This decrease in the total carbohydrate, contributed for the lactic acids production and pH reduction (Kakou *et al.*, 2010). On the contrary, the carbohydrate content reported during bulla fermentation was increase from 50% to 54 %.) (Taye Bezuneh 1984).

5.2. Approximate physicochemical analysis of ready to consume azo samples

The physico-chemical analysis of the ready to eat *azo* samples is shown on Table 5.2. The samples showed significant difference ($P < 0.05$) in carbohydrate, ash, protein, and fat contents, but not in pH and titratable acidity. The values in carbohydrate, ash, protein, and fat contents between the highest and lowest values showed a percentage difference of 35%, 26%, 22%, and 15%, respectively. This shows that there was quality difference amongst the *azo* products. This is in agreement with the Eyassu Seifu (2013) who observed significant variation on physicochemical composition *metata ayib*, a traditional Ethiopian fermented cottage cheese collected from different households. This may be due to the amount and kinds of spices used during preparation in different households.

Table 5.2: Results of Approximate physicochemical analysis of ready to consume *Azo* samples.

SC	Fat	Protein	Ash	Moisture	Carbohydrate	pH	TA
AZOI	3.75±0.07 ^a b	4.87±0.02 b	2.11±0.05 d	80.60±0.06 b	8.68±0.20 ^b	3.81±0.02 ^a	0.36±0.01 ^a
AZOII	3.54±0.02 ^c	5.04±0.08 ^a	2.35±0.04 b	77.17±0.09 c	11.9±0.23 a	3.80±0.01 ^a	0.35±0.02 ^a ^b
AZOIII	3.81±0.03 ^a	4.67±0.02 c	2.57±0.01 ^a	77.11±0.01 c	11.85±0.08 ^a	3.82±0.02 ^a	0.36±0.02 ^a
AZOIV	3.32±0.04 d	4.70±0.02 ^c	2.24±0.03 ^c	80.59±0.31 b	9.16±0.40 ^b	3.80±0.01 ^a	0.32±0.03 ^b
AZOV	3.66±0.09 bc	4.12±0.04 d	2.05±0.03 ^e d	81.48±0.06 a	8.70±0.22 ^b	3.81±0.02 ^a	0.34±0.01 ^{ab}

- SC=sample code.
- Values with the same superscript letter in a column are not significantly different ($P \leq 0.05$).
- Values with different superscripts in the columns differ significantly ($p > 0.05$).
- Values are means of duplicate determinations \pm SD.

The moisture content of *azo* samples at the time of consumption (AZOI and AZOII, AZOIII, AZOIV and AZOV) was within the range of 77.11 and 81.48% moisture. The protein content of ready to consume *azo* samples fell within 4.12% (AZOV) up to 5.04% (AZOII) showing a 22% difference between the lowest and highest value. However, the other samples did not show any significant difference in their protein content (Table 5.2). With regard to fat content, the different *azo* samples contained fat (3.32-3.81%,) with a significant difference in fat content (10-15%) between the sample lowest fat content in AZOIV (3.32%) and samples with the highest fat content. While, the fat content between AZOI and AZOII as well as AZOI and AZOV is insignificant ($P>0.05$) (Table 5.2). Likewise, there were significant variations in carbohydrate content among five *azo* samples at the time of consumption ranging from 8.04 to 10.89 (Table 5.2).

4.6. Microbial load of laboratory-prepared Azo

The mean log counts (CFU/g) of the dominant microbial groups of *azo* samples prepared in the laboratory are summarized in (Table 5.3). Counts of AMB, LAB, TC, mould and yeasts showed significant variation ($P<0.05$) but FC was found to be non-significant ($P>0.05$) at day 0 and 1. Salmonella was not detected at all during fermentation process.

AMB and LAB were dominant microflora during the fermentation process. The rapid increase in number of total viable counts of AMB and LAB was observed from day 0 to day 7 and the number slightly decreased after 15 and 30 days of fermentation (Table 5.3).

At initial (day 0) the count of AMB was 3.68 ± 0.05 log of CFU/g, which was exponentially increased until they reach maximum count at 6th day of fermentation 10.34 log of CFU/g. This was similar to the study done by Tetemke Mehari and Mogessie Ashenafi (1995) during *siljo*

fermentation, where AMB counts steadily increased from 4.77 to 10.88. On the contrary in awaze, high initial AMB count were reported (6.04 log of CFU/g) and decline after 2nd day of fermentation up to the end of fermentation (Ahmed Idris *et al.*, 2001). At first three days among the AMB isolates, the gram negative and gram-positive bacteria were proportional then after the Gram positive, oxidase negative and catalase negative organisms dominated gradually (data not shown). This finding is in agreement with the work of Ketema Bacha *et al.* 1998) who reported that at the start of *borde* fermentation dominate microorganisms in AMB count were Staphylococci, micrococci, members of Enterobacteriaceae, and Bacillus but as function of time increase dominated by Gram positive cocci and rods bacteria.

LAB also showed similar trend, their count increased from 3.35 to 10.49 CFU/g up to seven days of fermentation. Similar results also reported in siljo the LAB count increase with function of time day (day 0 to 5) from 1.78 to 10.82 log of CFU/g (Tetemke Mehari and Mogessie Ashenafi, 1995) and in awaze from 4.65 to 9.77 log of CFU/g from day 0 to day 4 respectively (Ahmed Idris *et al.*, 2001). The greatest increase in LAB count (above 2 log units) during this study was occurred between the 1st and the 2nd days of fermentation while in *borde* fermentation the greatest increase was reported with in 24h by 5 log units (kebede Abegaz, 2007).

Table5.3: Microbial count (log of CU/g) of Azo sample prepared in the laboratory

DF	AMB	LAB	Yeast and mold count			TC count	FC count
			Yeast (%)	Mould (%)	total count		
0	3.68±0.05 ⁱ	3.35±0.03 ^j	55.6	44.4	3.25±0.12 ^d	3.04±0.07 ^b	2.56±0.07 ^a
1	4.72±0.01 ^h	5.63±0.07 ⁱ	97	3	4.30±0.08 ^b	3.47±0.12 ^a	2.53±0.04 ^a
2	8.57±0.03 ^e	7.75±0.06 ^g	98.6	1.4	5.47±0.07 ^a	2.68±0.02 ^c	2.11±0.11 ^b
3	8.67±0.00 ^d	8.46±0.02 ^e	100	0	3.86±0.02 ^c	2.04±0.02 ^d	End
4	8.97±0.08 ^c	9.14±0.6 ^d	100	0	2.88±0.05 ^f	End	
5	9.39±0.30 ^b	9.69±0.01 ^c	0	0			
6	10.13±0.41 ^a	10.46±0.41 ^{ab}					
7	10.09±0.23 ^a	10.49±0.06 ^a					
15	7.49±0.04 ^f	8.33±0.01 ^f					
30	6.74±0.13 ^g	6.89±0.01 ^h					

- Values with the same superscript letter in a column are not significantly different ($P \leq 0.05$).
- Values with different superscripts in the columns differ significantly ($p > 0.05$).
- Values are means of triplicate determinations \pm SD
- DF=day of fermentation

In addition, the counts of yeast were tended to increase during the first 2 days of fermentation, followed steadily decreased until the 4th day of fermentation but after 5th day they reached to undetectable level. Similar results were reported on sour *Mifen*, a traditional fermented rice noodle from China in which the highest numbers of yeasts were observed on the first day ranged from 2.97 to 3.42 log of CFU/g, and then decreased to undetectable level on the third day (Han *et*

al., 2004). On the contrary, the growth of yeast in awaze were reported after 6th day of fermentation and increased gradually to reach the maximum count (6.40 log of CFU/g) at 14th days of fermentation. On other hand, yeasts were not detected throughout data fermentation (Ahmed Indris *et al.*, 2001).

Unlike yeast, the mould population was decreased as fermentation progressed. At initial mould represented 44% of total yeasts and mould counts in the PDA, which was the maximum count obtained during the fermentation and at day one and two of fermentation 3% and 1.4% of mould obtained respectively from total counts yeast and mould. Finally, no moulds were grown after the 3rd day of fermentation (Table5.3). Similar result also observed by Ketema Bacha (1998), the moulds count were declined from 4.40 log of CFU/g to undetectable level within 24h.

TC and FC were significantly decreased ($p < 0.05$) and then disappeared throughout the fermentation (Table 5.3). In the unfermented sample total, 3.04 ± 0.07 log of CFU/g of TC was observed and slightly increased to 3.47 after 1-day fermentation and later showed steadily decrease reaching to 2.04 log of CFU/g at day3. Finally, at 4th TC were reached undetectable level. Likewise, FC count tend to decrease from 2.68 to 2.11 ± 0.11 log of CFU/g from day 0 to day 2 and after 3rd day of fermentation they reached to undetectable level. The result found in agreement with the earlier report of Kebede Abegaz (2007), Ketema Bacha *et al.* (1998) on significant decrease in coliforms count of *borde* with increase in fermentation time. Ahmed Inders *et al.* (2001) also reported similar result; coliforms were detected only at the initial stages of '*datta*' fermentation then disappeared early during the fermentation

5.3. Comparative analysis of microbial load among *azo* samples

Azo from the five sources showed a total aerobic count in the range of 10.21 to 11.15 log of CFU/g and AZOIII coded sample was found to have the lowest AMB count (10.21 log of CFU/g); while, AZOII coded sample was found to have the highest count (11.15 log of CFU/g). Except for samples coded AZOI and AZOIV which were not significantly different ($P > 0.05$), the AMB count of Azo were showed significant variation ($P < 0.05$) in all samples. Furthermore, LAB counts were found in the range of 10.14 to 11.02 log of CFU/g. The LAB counts of AZOI, AZOIII, and AZOIV were statistically insignificant ($P > 0.05$), but the remained other samples were significantly varied ($P < 0.05$) with each other. However, moulds and yeast, TF, FC and salmonella were not detected in all samples.

Table5.4: Microbial counts (log of CFU/g) of laboratory and household prepared *azo* sample at the time of consumption.

SC	AMB	LAB
AZOI	10.35±0.04 ^c	10.15±0.13 ^c
AZOII	11.15±0.03 ^a	11.02±0.03 ^a
AZOIII	10.21±0.03 ^d	10.18±0.04 ^c
AZOIV	10.35±0.02 ^c	10.14±0.05 ^c
AZOV	10.59±0.13 ^b	10.42±0.03 ^b

- Values with the same superscript letter in a column are not significantly different ($P < 0.05$)
- Values with different superscripts in the columns differ significantly ($p > 0.05$)
- Values are means of triplicate determinations \pm SD

5.4. LAB Identification and Characterization

In total, forty two (42) selected strains were isolated from *azo* samples in which MRS agar was used as culturing medium. Of which twenty seven (27) were from laboratory prepared and the remaining fifteen (15) were from five ready to consume *azo* samples. After subsequent purification, these isolates were partially characterized based on Bergey's Manual of systematic bacteriology as done earlier by (Chowdhury, *et al.*, 2012; Mugula *et al.*, 2003; Nikita and Hemangi, 2012). Hence, all of isolates exhibited Gram-positive, oxidase negative, non-motile, no catalase activity and positive results for glucose fermentative ability all were confirmed as LAB.

In respect to colonies characteristic of the all isolates were appeared in creamy white elliptical, white mucoid on the surface and creamy white circular flat. The sizes of colonies were ranged from tiny to 3.5 mm in diameter (data not shown); this is in agreement with previous reports by (Uaboi-Egbenni *et al.*, 2009)

As shown in Table 5.5 after preliminary tests, the isolated LAB was subjected to different physiological and biochemical examination. It was found that, 26% isolates were capable of growing at 10⁰C and 15⁰C temperature of which the isolate 5% was also grew at 45⁰C. Likewise, other 21.4% isolates showed growth at both 15⁰C and 45⁰C but not grew at 10⁰C and 21.4% isolates grew only at 15⁰C and 33.3% isolate grew only at 45⁰C.

Table 5.5 Morphological, physiological and some biochemical characterization of LAB isolated during *azo* fermentation.

Characteristics	Percentage of isolate with positive reactions							
	Group	I	II	III	IV	V	VI	VII
	No. of isolates	4	9	13	9	4	1	2
Cell morphology		C	R & SR	R & SR	C	C	C	SR
CO ₂ from glucose		100	100	0	0	0	0	100
Arginine hydrolysis		0	100	NT	NT	NT	NT	0
Growth at temperature (C ⁰)	10	0	11	31	0	100	100	50
	15	75	67	92	0	100	100	100
	45	25	89	31	100	0	100	50
Growth at NaCl concentration (%)	2	100	100	100	100	100	100	100
	4	75	78	100	100	75	100	100
	6.5	50	22	31	100	25	100	100
	8	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0
Growth at pH	3.9	50	67	69	44	100	0	0
	9.6	100	56	46	67	75	100	100
Suspected genus		Leuconostoc	Lactobacillus	Lactobacillus	Pediococcus	Lactococcus	Enterococcus	Unidentified

❖ Whereas: C=cocci, R= rod SR= short rod and NT= not determined

In other cases, all isolates were grown in 2% NaCl concentration, 90.5% isolates were grown at 4% NaCl concentration of these 50% were also grown in 6.5% NaCl but the rest of 40.5 % isolates were only at 4% NaCl concentration. None of the isolates was grown at 8% and 10% NaCl concentration. In addition, 33.3% isolate were grown in pH 3.9 and 9.6; 28.6% isolates were grown only in 3.9; 31% isolate were grown only in 9.6 and 7 % isolates were grown neither in pH 3.9 nor 9.6.

Regarding to gas production from glucose, homofermentative accounted about 64%, heterofermentative 36%, while 21% of the heterofermentative isolates were able to hydrolyze arginine and appeared in rod and short rod shape but the rest 15% were not hydrolyze arginine; of these 10% were cocci and 5% were appeared in coccobacilli. From these homofermentative, 31% were characterized by rod and short rod shapes whereas 33% were cocci.

According to their similarities and difference presented in Table 5.5 LAB involved in Azo fermentation were divided in to 7 groups and tentatively identified as: *Leuconostoc* (Group I), heterofermentative *Lactobacillus* (Group II), homofermentative *Lactobacillus* (Group III), *Pediococcus* (Group IV), *Lactococcus* (Group V), *Enterococcus* Group VI and unidentified group as Group VII. The samples were dominated by the genus *Lactobacillus* with 52%, followed by the genus *Pediococcus* (21 %), *Leuconostoc* (10%) and *Lactococcus* (10%), unidentified group (5%) and *enterococcus* (2%).

Among 42 isolates, 4 (AZL5, AZL24, AZL41, AZL53 and AZL65) were categorized under Group I. Here, all isolates were isolated only in the early stages of fermentation (from day 0 to day 3) of laboratory prepared Azo sample (AZOI) (Table 5.6). However, high variability was observed on basis of their growth condition at different salt concentration, pH and temperature as

described in Table 5.6; they share some common characteristic to categorize under the same group. These include the only cocci produced CO₂ during glucose fermentation and shown negative result in arginine hydrolysis.

Accordingly, they were categorized under genus *Leuconostoc* (Nikita and Hemangi, 2012). In accordance with the results obtained in this study, Oyedeji *et al.* (2013) reported that the early stage fufu and Ogi fermentation Nigerian cassava root tuber and cereal base fermented foods were dominated by *Leuconostoc* then followed by *Lactobacillus*. Brhanu Abegaz Gashe (1987) also reported that *Leuconostoc mesenteroides* is initiated the fermentation of *kotcho*.

Isolates under group II and group III were the derivatives of genus *Lactobacillus* (Nikita and Hemangi, 2012), which were the most dominant LAB through Azo fermentation (Table 5.6); they consist of 22 isolates (52%) of LAB isolated from all samples. These isolates appeared in rod and short rod shape. Of these, 21% isolates were heterofermentative and able to hydrolyze arginine and the rest 31% isolates were homofermentative LAB, showed the negative result on gas production from glucose. This finding is similar to that reported by Tetemke Mehari and Mogessie Ashenafi (1995); Oyedeji *et al.* (2013) and Ogbonnaya and Chidinma (2012), who isolated *Lactobacillus* as predominant LAB from siljo, Nigerian traditional fermented food, *fufu*, *ogi* and *akamu* respectively. Similarly, report show that the plant based fermentation predominantly carried out by genus *Lactobacillus* of LAB (Mugula *et al.*, 2003). Furthermore, *Lactobacillus* was also the dominant LAB isolated during the study of traditional fermented goat milk in Indonesia (Yelnetty *et al.*, 2014).

Table 5.6 LAB distribution in azo fermentation

Sample code		Isolates	Dominating LAB genera during fermentation
AZOI	DF		
	0	AZL5 and AZL6	Leuconostoc and Lactobacillus
	1	AZL23 and AZL24	Pediococcus and Lactobacillus
	2	AZL40, AZL41 and AZL42	Pediococcus, Leuconostoc and Lactococcus
	3	AZL51, AZL52 and AZL53	Pediococcus, Lactobacillus and Leuconostoc
	4	AZL63, AZL64 and AZL65	Lactobacillus and unidentified
	5	AZL73, AZL74 and AZL75	Lactobacillus and Pediococcus
	6	AZL83, AZL84 and AZL85	Lactobacillus and Lactococcus
	7	AZL92, AZL93 and AZL94	Pediococcus, Lactococcus and Lactobacillus
	15	AZL99 and AZL100	Pediococcus
	30	AZL101 and AZL102	Lactobacillus
AZOII		AZH110, AZH111, AZH112 and AZH113	Lactobacillus and enterococcus
AZOIII		AZH115, AZH116 and AZH117	Lactobacillus, Pediococcus and unidentified
AZOIV		AZH122, AZH123, AZH124 and AZH125	Lactobacillus, Pediococcus and Lactococcus
AZOV		AZH127, AZH128 AZH132 and AZC133	Lactobacillus

Group IV were consists of nine isolates, they were cocci, homofermentative and the cell arrangement of most isolates were tetrad but occasionally they were also appeared in pair and short chain. The isolates in this group identified as *Pediococcus* the tetrad formation is the major characteristic to distinguish from other cocci isolates (Nikita and Hemangi, 2012; Yuliana and Dizon, 2011). This genus is the second largest LAB in this study. From the results, all *Pediococcus* able to grow in 6.5% NaCl concentration, 45⁰C temperature, 67%, and 44% in pH 9.6 and 3.9. Similar results reported by other researchers on involvement of the *Pediococcus* in several fermented foods for example, *borde* (Kebede Abegaz, 2007), *teff* dough (Berhanu Abegaz Gashesh, 1985); *kotcho* (Berhanu Abegaz Gashe 1987a) and fermented kapok and cassava (Kpikpi *et al.*, 2009).

Whereas, the four cocci isolates under group V were identified as *Lactococcus*. They grew at 10°C and 15°C but not at 45°C (Nikita and Hemangi, 2012). This result is supported by previous study by Senait Zewdie *et al* (1995) who isolated *Lactococcus* as one among other LAB genus in *siljo* fermentation and in Kunu-Zaki, Nigerian Sorghum based fermented beverage (Oluwajoba *et al.* (2013). According to the report by Kebede abegaz (2007) however, *Lactococcus* were not involved in *borde* fermentation.

Correspondingly, one isolate (AZH110) homofermentative cocci of Group VI were identified as genus *Enterococcus*. This isolate was isolated only in one household prepared Azo sample (AZOII) which differ from other cocci shaped isolates by its capability to grow in wide range temperature(10, 15 and 45⁰C), at pH 9.6 and 6.5 NaCl concentration which is an important characteristic for distinguishing the *Enterococcus*. Similar results were reported earlier by (Senait Zewdie *et al.*, 1995 and Berhanu Abegaz Gashe 1985) *Enterococcus* were isolated in *siljo* and *teff* dough.

The remaining two short rod isolates (AZL65 and AZH117) were grouped under group VII, unidentified group. Although, their capability to produce CO₂ glucose and shown negative result in arginine hydrolysis like *Leuconostoc*, further tests needed to differentiate whether they are *Leuconostoc* or *Weisselia*. According to Collins *et al.*, 1993 all *Weisselia* spp. are unable to grow in 45⁰C temperature (with the exception of *W. confusa*), therefore, AZH117 most likely could be *Leuconostoc* if it is not *W. confusa* because it able to grow in 45⁰C. However, AZL65 were grown only at 10⁰ C and 15⁰ C, therefore, it could be either of them.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. CONCLUSION

The results for proximate physicochemical composition and microbial load of *azo* samples showed that fermentation increased the quality of the food in three aspects. First, improve nutritional status by increasing protein content from 3.28 to 4.87 and reduction of fat content from 4.74 to 2.59. Second, the process reduced total and fecal coliforms the indicative organisms for the presence of pathogens and reduction of food spoiling organisms such as molds. The growth of lactic acid bacteria in the food considered as one factor contributes to the elimination of undesirable microbes. The result in this finding also indicated that lactic acid bacteria in which *Lactobacillus* and *Pediococcus* were dominant microorganisms carried out the fermentation.

6.2. RECOMMENDATIONS

Based on the results of this study, it is possible to provide the following points as recommendation:

- ❖ More detail investigation should be conducted on the role of LAB nutritional and health value of the food.
- ❖ Endod is presumed to contain toxins and other alkaloids, and the role of fermentation to detoxify these toxins should be studied in the future.
- ❖ Further study also needed toward the chemo-toxicity of the fermented food to be sure for its quality.

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APPENDIX

Appendix 1: Table showing the physiological and biochemical tastes to characterize LAB

isolates	cell Morphology		Gas from glucose	Arginine hydrolyze	Growth Temperature (C ⁰)			Growth NaCl %					Growth at pH	
	Arrangement	Shape			10	15	45	2	4	6.5	8	10	3.9	9.6
AZL5	Pair and Chain	C	+	-	-	+	-	+	-	-	-	-	+	+
AZL6	Chain	SR	-	NT	+	+	-	+	+	-	-	-	-	+
AZL23	Pair, Tetrad and short Chain	C	-	NT	-	-	+	+	+	+	-	-	-	+
AZL24	Pair and chain	C	+	-	-	+	-	+	+	+	-	-	+	+
AZL40	Pair and tetrads	C	-	NT	-	-	+	+	+	+	-	-	-	+
AZL41	Pair and chain	C	+	-	-	+	-	+	+	-	-	-	-	+
AZL42	Chain	C	-	NT	+	+	-	+	+	+	-	-	+	-
AZL51	Pair and Tetrad and short chain	C	-	NT	-	-	+	+	+	+	-	-	+	+
AZL52	Single and Pair	R	+	+	-	+	+	+	+	-	-	-	-	+
AZL53	Pair and chain	C	+	-	-	-	+	+	+	+	-	-	-	+
AZL63	Single & chain	SR	-	NT	-	+	-	+	+	+	-	-	-	-
AZL64	Single, pair & chain	SR	+	+	-	+	+	+	+	-	-	-	-	+
AZL65	Single, pair and chain	SR	+	-	+	+	-	+	+	+	-	-	-	+
AZL73	Chain	SR	-	NT	-	+	-	+	+	-	-	-	+	-

AZL74	Chain	R	+	+	+	+	-	+	+	-	-	-	+	-
AZL75	Pair and Tetrad and short chain	C	-	NT	-	-	+	+	+	+	-	-	+	+
AZL83	Pair	C	-	NT	+	+	-	+	-	-	-	-	+	+
AZL84	Short chain	R	-	NT	-	+	-	+	+	-	-	-	+	+
AZL85	Pair and chain	R	+	+	-	-	+	+	+	+	-	-	+	+
AZL91	Pair and tetrad	C	-	NT	-	-	+	+	+	+	-	-	-	-
AZL92	Chain	C	-	NT	+	+	-	+	+	-	-	-	+	+
AZL93	Chain	SR	-	NT	-	+	-	+	+	-	-	-	+	-
AZL94	Pair and chain	SR	+	+	-	-	+	+	+	-	-	-	-	-
AZL99	Pair and tetrads	C	-	NT	-	-	+	+	+	+	-	-	-	+
AZL100	Tetrads	C	-	NT	-	-	+	+	+	+	-	-	-	+
AZL101	Chain	SR	-	NT	+	+	+	+	+	+	-	-	-	+
AZL102	Single	SR	-	NT	-	-	+	+	+	+	-	-	+	-
AZH110	Chain	C	-	NT	+	+	+	+	+	+	-	-	-	+
AZH111	Single, pair and chain	SR	-	NT	-	+	+	+	+	+	-	-	+	+
AZH112	Chain	R	-	NT	-	+	+	+	+	+	-	-	+	-
AZH115	Pair, Tetrads &short chain	C	-	NT	-	-	+	+	+	+	-	-	+	-
AZH116	Chain	SR	-	NT	-	+	-	+	+	-	-	-	+	-
AZH117	single, pair and chain	SR	+	-	-	+	+	+	+	+	-	-	-	+
AZH122	Chain	R	+	+	-	-	+	+	+	+	-	-	+	+
AZH123	Single, pair and	C	-	NT	+	+	-	+	+	-	-	-	+	+

	chain													
AZH124	Single	R	+	+	-	+	+	+	+	-	-	-	+	+
AZH125	Tetrads and short chain	C	-	NT	-	-	+	+	+	+	-	-	+	-
AZH127	Chain	SR	-	NT	-	+	-	+	+	-	-	-	+	+
AZH128	Single and chain	R	+	+	-	+	+	+	+	-	-	-	+	-
AZH132	Single, pair & chain	R	+	+	-	+	+	+	+	-	-	-	+	-
AZC133	Single and short chain	R	-	NT	+	+	-	+	+	-	-	-	+	-
AZC134	Single, pair & chain	SR	-	NT	+	+	-	+	+	-	-	-	+	+

Whereas: + = positive, - = negative, C= cocci, NT= not determined, R=rod, SR=short rod

