

**ADDIS ABABA UNIVERSITY**  
**SCHOOL OF GRADUATE STUDIES**  
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**EVALUATION OF MICROBES FROM *TEFF* (*Eragrostis tef* (Zucc. ) Trotter)**

***ERSHO* FOR *INJERA* STARTER CULTURE DEVELOPMENT**

**BY**  
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**Evaluation of microbes from *Teff (Eragrostis tef (Zucc. ) Trotter) Ersho* for  
*Injera* Starter Culture Development**

**MSc. Thesis**

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## **DECLARATION**

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any other University, and that all sources of information used for the thesis have been duly acknowledged.

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## **Abbreviations**

AAB	Acetic acid bacteria
AMB	Aerobic mesophilic bacteria
CMC	Carboxyl methyl cellulose
DNA	Deoxyribonucleic Acid
EIAR	Ethiopian institute of agricultural research
LAB	Lactic acid bacteria
HCL	Hydrochloric acid
H <sub>2</sub> S	Hydrogen sulfide
ITS	Internal transcribed spacer
MRS	de Man Rogosa and Sharp
NCBI	National center for biotechnology information
NABRC	National agricultural biotechnology research center
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PH	Power of hydrogen
SPSS	Statistical package for social sciences

VRBA	Violet red bile agar
YPD	Yeast extracts peptone dextros
TSA	Triple sugar iron agar

## Abstract

*Injera* is fermented Ethiopian ethnic traditional staple food prepared usually from tef (*Eragrostis tef* (Zucc.) Trotter). The *teff injera* is prepared at households by mixing *teff* flour with water and using a starter (*ersho*) which is left over from the last fermentation. The batter is fermented using the back-slopping technique which involves adding *ersho*. The microbial composition of *ersho* is not defined. Therefore, this study was undertaken to isolate, screen, and identify fermentative microbes from *ersho* and their potential for starter culture development. A ninety-one *ersho* samples were collected from Debrebirhan, Fiche, Chacha, Sebata, Teji, Holeta, Adama, Bishoftu, and Dukam. Lactic acid bacteria, aerobic mesophilic bacteria, yeasts, and molds were isolated from the *ersho* samples collected. Isolated microbes were screened for their sugar utilization stress test, temperature stress test, pH tolerance test and hydrogen sulfide production test. The best performing isolates were used to formulate the starter culture for *injera* fermentation by combining the four microbial groups. The sensory acceptability of the selected *injera* scored highest mean of other, was produced from a combination of T4 (L1+L2+Y1+Y2+AMB+M) and fermented for 28 hrs. Finally, based on DNA sequencing, bacteria identified as *Bacillus subtilis*, *Lactocaseibacillus paracasei* and *bacterium strain AGE YJ E2*. The yeast and mold belong to the *Pichia fermentans* strain and the *Aspergillus niger* strain, respectively. The selected starter culture requires further analysis of its profile, performance, and shelf life.

**Keywords;** Aerobic mesophilic bacteria, *ersho*, fermentation, lactic acid bacteria, mould, starter culture and yeast

## 1. Introduction

Fermentation is one of the ancient and most affordable processes for producing and preserving food (Blandino *et al.*, 2003). Fermented foods are generally defined as foods or drinks processed through controlled microbial growth and enzymatic modifications of primary and secondary components of foods (Marco *et al.*, 2017). Primary and secondary metabolites, including antibiotics, carbon dioxide, alcohol, vitamins, folates, and organic acids, are created during the fermentation process (Marco *et al.*, 2017). Through the enzymatic action of microorganisms, primarily yeasts, bacteria, and molds, organic molecules are broken down into acids or alcohol in either aerobic or anaerobic conditions (Chilton *et al.*, 2015).

Different methods, ingredients, and microbes are used to make different kinds of fermented food and beverage products (Mulaw and Tesfaye, 2017). Alkali, lactic acid, acetic acid, and alcoholic fermentation are the four primary categories of fermentation. Lactic acid bacteria (LAB) are the primary organisms responsible for lactic acid fermentation. Some examples include Cereal-based fermented foods, kimchi, sauerkraut, *injera*, thainham (fermented fresh pork) and fermented milks (Steinkraus, 2002). In alcoholic fermentation, yeasts produce ethanol. Examples of alcoholic fermentation include wines, beers, vodka, whiskey, brandy, and bread. In Africa, Ethiopian *tej* and *talla*, Kenyan muratina and busaa, Nigerian pito, Zambian maize beer are well known (Steinkraus, 2002). *Acetobacter species* are responsible for acetic acid fermentation. *Acetobacter* converts transforms alcohol into acetic acid in the presence of oxygen (e.g., vinegar) (Anal, 2019). Alkaline fermentation takes place during the fermentation of soybeans, fish, and seeds, popularly used as a condiment (Anal, 2019). Generally, an alkaline fermentation involves molds in the process.

Africa is home to the widest range of fermented foods, which are staples in the diets of its people and have a significant impact on social, economic, and health conditions.. Ethiopia is one of those countries, where a wide range of traditional fermented foods and drinks are made and consumed. (Wedajo, 2020). These include fermented plant products (*injera*, *kocho*, etc.), fermented milk products (*ergo*, *ayib*, etc), fermented beverages (*tella*, *tej*, *shamita*, etc.), and fermented condiments (*awaze*, *data*, etc.) (Ashenafi, 2006).

Ethiopian fermented foods and beverages are made via acid-alcohol fermentation (Wedajo, 2020). *Injera*, a commonly used and traditionally prepared fermented food made from a raw material called *teff*, is one of many forms of fermented indigenous food. *Teff* (*Eragrostis tef* (Zucc) Trotter) is a tropical cereal that is widely grown as a grain crop in Ethiopia (Bultosa *et al.*, 2002). It is the preferred seed for producing *injera* (a soft, spongy, sour circular flatbread with honey comb on the upper surface), which is a staple dish for the majority of Ethiopians (Bultosa *et al.*, 2002; Bultosa *et al.*, 2008 and Abebe and Ronda, 2014). *Injera* is made from flour, water and seed culture called *ersho*. *Erscho* is a liquid that was kept from previously fermented dough (Hassen *et al.*, 2018). The two stages of natural fermentation involved in the preparation of *teff injera*, take one to three days on ambient temperatures (Mulaw and Tesfaye, 2017). After the primary fermentation is ended, a portion of the batter mixed and boiled to make *absit*. After preparing the *absit*, it mixed back to the original fermented batter and let to undergo secondary fermentation for one to two hours. Finally, the batter is ready for *injera* preparations (Gebrekidan and Gebrehiwot, 1982).

The microorganisms responsible for fermentation in indigenous fermented foods are usually the microflora naturally contributed from the raw substrate, utensils and environment (Attuquayefio, 2014). Different workers in the area indicated the following microbes to be involved in *injera* fermentation. During *injera* dough fermentation members of the *enterobacteriaceae* (*Enterobacter* spp., *Escherichia coli*, *Klebsiella* sp., *Proteus* sp., and *Citrobacter* sp.) initiated the fermentation and their activities reduced the pH of the dough to about 5.8 during the first eighteen hours of fermentation (Berhanu *et al.*, 1982). The pH is then further decreased by lactic acid bacteria (*Pediococcus pentosaceus*, *Lactobacillus fermentum*, *Lactococcus piscium*, *Lc. plantarum*, *Ped.acidilactici*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Lc. raffinolactis*, *Leuc. mesenteroides* subsp. *dextranicum*, and *Enterococcus cassiiflavus*) to 4.7 at (18 - 72 hrs) (Dandessa, 2019). The dough acidic feature is caused by the lactic acid bacteria. Yeasts only show up in significant numbers at a later stage of the fermentation (Gashe, 1985) and shown to include *Pichia fermentans*, *P. occidentalis*, *Candida humilis*, *Saccharomyces cerevisiae*, and *Kazachstania bulderi* were shown by (Tadesse *et al.*, 2019).

In order to make *injera*, a starter (*Ersho*) saved from the previous fermented batter and used to start new batches of fermentation (back slopping) (Bultosa *et al.*, 2008; Desiye and Abegaz, 2013). Wedajo (2020) showed that it has 96.4 % moisture, 0.05 mg of riboflavin per 100 gm and 0.4 mg of niacin per 100 gm. According to Ashenafi (1994) work, the following microorganisms to be involved in *ersho*: the members of aerobic mesophilic bacteria and this consisted of only *Bacillus* spores. Lactic acid bacteria species were *Pedococcus cerevisiae*, *Lactobacillus brevis*, *Lactobacillus fermentum* became the dominant flora. The yeast species were *Candida millera*, *Rhodotorula mucilaginosa*, *Kluyveromyces marxianus* and *Pichia naganishii* (Mogessie, 1994). They may be important in leavening the batter of *teff* and producing flavor compounds in the later stages of fermentation (Ashenafi, 2002).

Traditional food fermentation processes have always depended on spontaneous fermentation. The growth of the microbiota that naturally exists in the fermentable raw material (Leroy and De Vuyst, 2004; Civas-Limon *et al.*, 2020). The outcome of such processes is unpredictable because the composition and microbial load of the sources determine the inoculum. The back-slopping technique can be seen as the earliest advancement of such spontaneous processes (Nout, 1994). Back-slopping is the process of introducing a small portion of a previously completed successful fermentation into the fermentable raw material. Generally, spontaneous fermentation is carried out by microbes found in raw food ingredients or those involved in the backslopping process which entails inoculating a fresh batter with a small quantity of a previously fermented batch. Furthermore, inefficiency, low product yields, and unpredictable product quality might result from natural fermentation (Jay, 2000). A breakthrough in the processing of fermented foods has been the addition of specific starter cultures to raw materials, which has resulted in a high degree of control over the fermentation process and standardization of the end product (Leroy and De Vuyst, 2004). As a result, the goal of this research was to isolation and characterization of microbes from *teff ersho* and to formulate and develop a potential starter culture for a large scale production of *injera*.

## 1.2. Statement of the problem

Microbes play an important role by altering the physical and nutritional properties in traditional fermented food products made from plant and animal sources (Keyeta, 2021). Traditional practices are used in Ethiopia for the preparation and consumption of *injera* (Neela and Fanta, 2020). The use of appropriate starter cultures improving the fermentation process, facilitates the control over the early stages of fermentation and the predictability of the outcome of derivative goods also lowers the microbiological instability and organoleptic variability of fermented foods (Kimaryo *et al.*, 2000; Holzapfel, 2002). Controlled fermentation can be achieved by use of pure or mixed starter cultures with appropriate technology (Glover *et al.*, 2009).

Back-slopping or adding *ersho* are the main ways that microorganisms introduced into the batter to initiate the fermentation process that produces *injera*. The microbial composition of *ersho* is not defined and depend on the age, pH, nutrient content, and storage condition. This is one of the factor that makes the final product quality unpredictable and inconsistent (Kimaryo *et al.*, 2000). Thus, the traditional processes needs to be transformed into a new methods that integrates novel ways of process, control and optimization, as well as a standardization of raw material and final product product quality without losing their desirable attributes (Cencic and Chingwaru, 2010). Therefore, the goal of the current work was to isolate and characterize the microbes from *ersho* and to develop a potential starter culture for a large scale production of *injera*.

### **1.3. Objectives**

#### **1.3.1. General objective**

- The general objective of this study was to isolate and characterize fermentative microbes from *ersho* samples and to develop microbial starter culture for large scale production of standardized *teff injera* with consistent quality and acceptance.

#### **1.3.2. Specific objectives**

The specific objectives of the current study were:

- ❖ To isolate, screen, characterize and identify the best fermentative & representative microbes from *ersho* samples.
- ❖ Formulating starter cultures from the selected best representative microbial isolates & produce *injera*
- ❖ Evaluating the sensory quality of the ready-to-consume *injera* with selected starter formulations
- ❖ Identifying the isolates included in the final selected starter formulate at molecular level

## **2. Literature review**

### **2.1. Overview of fermentation**

Historically, fermentation is among the earliest ways of food processing and preservation that has been evolved by default rather than by design (Stiles, 1996). For thousands of years, there has been marked rise in the demand for fermented foods, both in terms of production and consumption. As a result, these foods have become a substantial part of the worldwide diet. More than anything else, man has been using microbes to prepare food products for thousands of years, and a wide range of fermented foods and drinks have made a substantial nutritional contribution to the diets of many people throughout the world (Achi, 2005). Fermentation is a biotechnology that promotes and controls the growth of microorganisms and their metabolic activities for the preservation and transformation of raw food material (Mcneil *et al.*, 2013; Ravyts *et al.*, 2012). These processes are characterized by low energy requirements, which enable microbial fermentations to continue without external heat sources (Achi, 2005).

Fermentation is the process by which microbes convert carbohydrates into alcohols, carbon dioxide, and/or organic acids under largely anaerobic circumstances in order to produce energy. (McGovern *et al.*, 2004). Fermentation is food preservation technique that reduces the rate of spoilage and produces foods with a longer shelf life by accumulating alcohol and organic acids and increasing the acidity of food substrates. This inhibits the growth of other microorganisms and the activity of enzymes in the food system. Additionally, it is employed to improve the organoleptic qualities (e.g., taste and texture), with some foods, such as olives, being inedible without fermentation which gets rid of bitter phenolic compounds (Dimidi *et al.*, 2019).

Microorganisms are crucial to the human diet because they alter the physical, chemical, and nutritional properties of fermented foods derived from both plant and animal sources (Christoph *et al.*, 2017). Furthermore, they have a favorable impact on the health-promoting value of food due to the presence of probiotic microorganisms and increasing microbial safety (Admassie, 2018; Yu *et al.*, 2021). During a fermentation process, the metabolites of the microorganisms involved limit the formation of undesirable chemicals and the development of unwanted microflora (Śmiechowska *et al.*, 2018).

## 2.2. The beginning of fermented foods

The term “fermentation” is borrowed from the Latin word *fevere* which means “to boil.” According to Louis Pasteur, fermentation was defined as “*La vie sans l’air*”, i.e., life without air, and the science of fermentation is also known as zymology or zymurgy. The art of food fermentation dates back to prehistoric times and are the oldest methods for producing new foods from existing substrates, and of prolonging the shelf life of foods (Okpara and Ugwuanyi, 2017; Mishra *et al.*, 2017).

According to Prajapati and Nair (2003), fermentation historically began in the Indian subcontinent in the settlements that existed before the renowned Indus valley civilization. In the fertile crescent of Iraq between the Tigris and Euphrates rivers, the art of making cheese dates back to 8000 years ago, when domestication of plants and animals was just being domesticated (Fox, 1993). Later, it is believed that the Egyptians and Sumerians invented the alcoholic fermentations used in winemaking and brewing between 4000 and 2000 BC. Dough fermentations were also invented by the Egyptians, who employed them to produce leavened breads as early as 4000–3500 BC (Prajapati and Nair, 2003).

Depending on the microorganism, fermentation produces different end products. With Leeuwenhoek and Hooke naming and identification of microorganisms (in 1665) came the scientific rationale for fermentation (Gest *et al.*, 2004). Pasteur then used carefully planned experiments to demolish the "spontaneous generation theory" in 1859 (Farley and Geison., 1974). Sir Joseph Lister investigated the activity of a unique bacterium called *Lactococcus lactis* in fermented milk in 1877 (Santer, 2010). Generally, fermentation is mostly caused by microbes that exist as individual cells or groups of cells. These microbes are typically bacteria, occasionally fungus, or cells derived from plants or animals. In 2002, Members of the IDF Task force created an updated inventory of microorganisms (fungus, bacteria, filamentous fungi, and yeasts) utilized in food fermentations encompassing a range of food matrices (Bourdichon *et al.*, 2012).

### 2.3. Types of fermentation

Fermented food products are made widely using variety of methods, microbes and raw materials. However, there are basically only four types of fermentation processes namely, alcoholic, acetic acid, lactic acid, and alkali fermentation are used in the process of developing new products (Soni and Sandhu, 1990). Fermentations are carried out by a numerous microorganisms: yeast is typically involved in alcoholic fermentations; *Bacillus* spp. are typically involved in alkaline fermented foods; lactic acid bacteria are most frequently found in fermentations where lactic acid is the end product; moulds are involved in the fermentation of legumes and cheeses (Johansen, 2018).

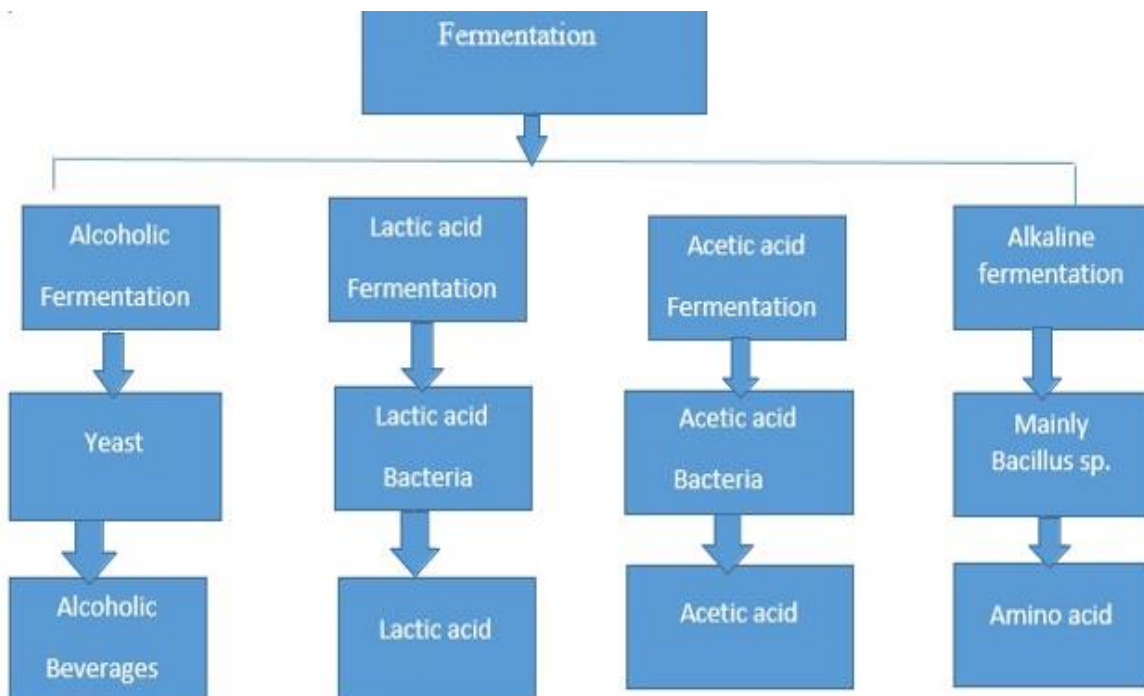


Figure 1. Schematic representation of types of fermentation, the microorganisms involved, and the resulting end products (Anal, 2019).

#### 2.3.1. Alcoholic fermentation

Alcoholic fermentation has been practiced since ancient times and it's one of the most significant and ancient food processing methods. This technique uses yeast or occasionally yeast-like molds like *Amylomyces rouxii*, mold-like yeasts, like *Endomycopsis*, and bacteria like *Zymomonas mobilis*, to produce a variety of alcoholic drinks, such as wine, distilled liquors, and beers. By

using fermentable sugars from substrates including cereal grains, sugar cane juice, fruit juices, palm sap, diluted honey, or hydrolyzed starch, these microbes produce mostly carbon dioxide and ethanol (Anal, 2019).

### **2.3.2. Lactic acid fermentation**

Lactic acid bacteria (LAB) are a type of gram-positive bacteria that use carbohydrates primary or exclusive carbon source (George *et al.*, 2018). Lactic acid bacteria are generally cocci or rods, and are highly tolerant to low pH. There are more than 60 genera of lactic acid bacteria; *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Enterococcus*, *Weissella*, and other genera are commonly discovered during food fermentation (Mokoena, 2017). In fermented food processing, the degradation of polysaccharides by lactic acid bacteria can produce monosaccharides or lactic acid, etc., which can improve the quality of food (Wang *et al.*, 2021). Many essential metabolic characteristics should be possessed by lactic acid bacteria, such as the capacity to hydrolyze to hydrolyze protein, the ability to produce viscous exopolysaccharides and the ability to inhibit bacteria (Wang *et al.*, 2021). These compounds result in the enhancement of shelf life and microbial safety, as well as the improvement of texture and sensory profile of the fermented products (Anal, 2019). Fermented dairy products (cheese, butter, and butter milk, yoghurt, fermented probiotic milk, kefir), fermented meat products (sausages), fermented fish products, fermented fresh vegetables (kimchi, cabbage, pickles), fermented cereals (sourdough bread and bread-like products), and alcoholic beverages (wine) are among the various fermented products that are available, most of which are produced by lactic acid fermentation (Frédéric and De Vuyst, 2004).

### **2.3.3. Acetic acid fermentation**

Acetic acid bacteria (AAB) are commonly found in a wide range of fermented foods and beverages. According to Gomes *et al.*, (2018), these microorganisms are gram-negative, strictly aerobic, and catalase-positive. The AAB belong to the family Acetobacteraceae: they are currently classified in 19 genera (*Acetobacter*, *Acidomonas*, *Ameyamaea*, *Asaia*, *Bombella*, *Commensalibacter*, *Endobacter*, *Gluconacetobacter*, *Gluconobacter*, *Granulibacter*, *Kozakia*, *Neoasaia*, *Neokomagataea*, *Nguyenibacter*, *Saccharibacter*, *Swaminathania*, *Swingsia*, and *Tanticharoenia*) (Trček and Barja, 2014) and 92 species having been discovered to date (Qiu, Zhang and Hong, 2021).

AAB is present in fermented foods and beverages such as kombucha, milk kefir, water kefir, and acidic beers (Jayabalan *et al.*, 2014). AAB are predominantly known for their use in the production of vinegar, vitamin C, and cellulose. The food substrate rich in carbohydrates, sugar, alcohols, and/or ethanol enables AAB to rapidly and incompletely oxidize these substrates into organic acids (acetic acid) (Roos and Vuyst, 2018). However, AAB are not studied to the same extent as many other foodgrade and industrially important microorganisms. Furthermore, AAB are regarded as undesirable spoilers in the fermentation of alcohol (Dutta and Gachhui, 2007).

#### **2.3.4. Alkaline fermentation**

Alkaline fermented food products play a crucial part in the diet of people from Asia, Africa, and the rest of the world. Protein-rich foods are the main substrate that are acted upon primarily by bacillus spp., but other secondary microorganisms such as LAB, Staphylococci, and Micrococci are also involved (Anal, 2019). Alkaline fermentation involves Proteolysis, a process involved in alkaline fermentation, releases important amino acids and peptides. They are made with a variety of raw ingredients, such as fish species, African locust beans, and soybeans (Okpara and Ugwuanyi, 2017). Alkaline fermentation often takes place during the fermentation of fish and seeds, popularly used as condiments (Blandino *et al.*, 2003). Moreover, amino acids undergo degradation to produce ammonia and other alkaline molecules that raise pH (8–10). Alkaline-fermented foods include soumbala, ugha, bikalga, and ntoba mbodi from Africa; as well as kinema, natto, and thua nao from Asia (Ouoba, 2017). *Bacillus amyloliquefaciens*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. subtilis*, *B. subtilis var. natto*, and *B. Thuringiensis* are among the species of bacillus that are commonly found in legume-based fermented foods. whereas strains of *B. cereus* have been isolated from the fermentation of *Prosopis africana* seeds used in Nigeria to produce okpehe (Anal, 2019).

#### **2.4. An overview of African fermented foods**

Traditional fermented foods still rely heavily in the diet of numerous societies worldwide. African foods include fermented and non-fermented sorghum, maize, millet, and cassava products, as well as wild legume seeds and tubers, in addition to meat, dairy, and alcoholic beverages (Tamang and Samuel, 2010). According to Olasupo *et al.*, (2004) traditional food fermentation is significant to Africans because it enhances the the flavors of basic foods like grains and root crops, preserves

food at a low cost, and increases the digestibility and nutritional content of raw foods. The wide range of locally produced fermented foods and beverages across Africa results from a wide variety of fermentation types (lactic acid, alcohol, alkaline fermentation), substrates, microorganisms, and methods (Obafemi *et al.*, 2022).

While spontaneous fermentation is the predominant method of food fermentation in Africa, there are also examples where the microorganisms were introduced by back-slopping from previously fermented foods into newly fermented foods (Mokoen *et al.*, 2016). African fermented foods have been reported to be sources of many potentially nutritionally beneficial and/or health promoting components, including microorganisms (Obafemi *et al.*, 2022). Probably the most ancient and well-known among Africans (Dirar, 1993), lactic acid fermentation is primarily a home-based activity that is practiced all over the continent (Oyewole, 1997).

## **2.5. Commonly consumed African fermented foods**

Around the world, a wide different types of fermented foods are consumed. Every country has a unique variety of fermented foods, representing the staple diet and the raw ingredients available in that particular place (Campbell-Platt, 1987). In order to combat poverty, malnutrition, and hunger among African consumers, fermented foods are crucial (Fagunwa and Olanbiwoninu, 2020). The wide variety of African diets includes a substantial amount of fermented foods; certain foods are categorized as beverages, sauces, or staple foods, for example (Obafemi *et al.*, 2022). Foods can also be divided into groups according on the ingredients used in their preparation. These include products made fro tubers (e.g., *Fufu*, *Lafun*, and *Gari*); cereal-based products (e.g., *Ogi*, *Burukutu*, *Kunnu-zaki*, *Pito Humulur* and *Hussuwa*); legume-based products (e.g. *Dawadawa*, *Ogiri*, and *Ugba*); beverage-based products (e.g., *Emu*, *Oguro*), and dairybased products (e.g., *Fura*, *Nunu* and *Wara-Kishi*) (Obafemi *et al.*, 2022). While camel's milk is primarily fermented in Northern Africa and the Sudan region, milk from cattle, sheep, and goats are commonly fermented in Eastern and Southern Africa, as well as some regions in North and (less frequently) West Africa, where keeping such livestock has a long tradition.

Fermented starchy roots, primarily made from cassava (*Manihot esculenta* Crantz), are typical of West African cuisine, however other fermentations of cassava are also found throughout the continent (Okafor and Ejiolor, 1990; Kimaryo *et al.*, 2000; Ray and Sivakumar, 2009). Specific to Ethiopia is the *enset* (*Ensete ventricosum*) (also called false banana, Ethiopian banana, or Abyssinian banana) plant, which, can be transformed into “kocho” by lactic fermentation (Gashe, 1987). Meanwhile, *teff* (*Eragrostis tef*), which is also native to Ethiopia, is used to make the traditional sourdough-like, acid-leavened pancake known as *injera* (Oyewole, 1997). In Africa, the most popular fermented foods are millet, sorghum, and maize (corn). Rice and wheat are infrequently utilized in traditional food fermentations in Africa, in contrast to Asia and Europe (Oyewole, 1997). In Africa, fermenting foods made of cereals is a common method of food preservation. It is a low-tech, home-based system that has fed millions of people. Nowadays, a variety of fermented foods are produced from cereals at house hold and semi industrial scale. These foods are utilized for both adult consumption and as weaning meals for newborns and children (Lei and Jakobsen, 2004; Kalui *et al.*, 2008).

### **2.5.1. Fermented cereal products**

Cereals, which are mostly made by natural fermentation, account for up to 80% of the total calories consumed in several African nations. They are also a major source of fiber, vitamins, minerals, proteins, and carbs for people worldwide (Chavan and Kadam, 1989). The major cereal-based foods are wheat, rice, sorghum, millet, and maize. The fermented cereal dishes have two different textures: liquid (porridge) and stiff gels (solid). Granola porridges (gruels) contain things like ogi, mahewu, and mawe; cereal gels include things like kenkey, kiswa, and *injera* (Osungbaro, 2009). Fermented foods made from cereals are thought to be high in micronutrients and beneficial bacteria, and they are considered to be foods that promote health (Achi and Ukwuru, 2015; Fuller *et al.*, 2016; Okafor *et al.*, 2018).

Food is preserved and has improved organoleptic properties as a result of lactic acid bacteria producing lactic acid and other metabolites (Guyot, 2012). These LABs include *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum*, *Levilactobacillus brevis*, *Lactococcus lactis*, *Leuconostoc* species, and *Pedicoccus* species, while the other bacteria represented include *Streptococcus* and *Corynebacterium* species. Yeasts such as *Saccharomyces cerevisiae*, *Geotrichum fermentum*, *Candida tropicalis*, *Rhodotorula graminis*, and molds such as *Aspergillus*

*species, Fusarium subglutinans, Rhizopus nigricans, and Penicillium citrinum* (Ezekiel *et al.*, 2019; Achi and Asamudo, 2019; Adesulu-Dahunsi *et al.*, 2018).

## **2.6. Ethiopian traditional fermented food**

Traditional fermented food is an important part of the diet and cultural heritage that has been widely consumed since ancient times (Kavitake *et al.*, 2018). Traditional foods and beverages are natural diets or drinks that our ancestors were given throughout human evolution, particularly in the prehistory of food and beverage industrialization. Ethiopian traditional foods and drinks have a rich history that goes back to the earliest days of agricultural innovation and crop domestication. They are connected to a number of celebrations such as holidays, festivals, social gatherings, memorial services, and unique events (Anteneh and Tetemke, 2011; Steinkraus, 1983). For a very long time, the nation has relied on traditional foods and drinks for a variety of purposes, including socioeconomic advantages, medicine, nutrition, and guaranteeing food security.

In Ethiopia many different types of traditional fermented foods and drinks are prepared and consumed. Fermented foods derived from plants and animals are essential to human nutrition because bacteria change the physical, chemical, and nutritional properties of food (Christoph *et al.*, 2017). Ethiopian local fermented foods and drinks are made by an acid-alcohol fermentation process. These comprise the following: *injera, ergo, ititu, ayib, qibe, arrera, kocho, tella, siljo, datta, awaze, borde, tej, areki, cheka, azo, keribo, and korefe*. Of these *tella, cheka, tej, borde, areki, keribo, and korefe* are among the varieties of fermented beverages widely consumed in Ethiopia. On the other hand, traditional fermented condiments such as *awaze, datta, siljo, and azo* are also well-known in Ethiopia (Wedajo, 2020).

## **2.7. Injera fermentation**

*Injera* is a classic Ethiopian and Eritrean ethnic staple dish that is also popular in Somalia (Godebo and Niguse, 2019). Ethiopian food is centered around *injera*, a fermented pancake-like bread that is made from a range of cereals, such as *teff*, wheat, barley, sorghum, or maize, or a combination of several of these cereals, depending on availability and quantity (Umer, 2019), but *teff* (*Eragrostis tef*) is the main cereal ingredient in Ethiopian *injera*. *Teff* is commonly used to make a variety of drinks and foods, like gruel, porridge, and *injera* (leavened pancakes) and *kita* (unleavened pancakes) (Ebba, 1996).

Due to its gluten-free nature and the easily digestible nature of most of its prolamins (Schneider and Anderson, 2010), *teff* is a good alternative to wheat for celiac disease and gluten-free diets. Compared to other cereal grains, it has a higher nutritional value since it has more minerals (primarily iron, calcium, phosphorus, and copper) and all the essential amino acid composition, including lysine. It is high in fiber, which can benefit diabetic patients and includes the B1 vitamin (Umeta and Parker, 1996). *Teff* is tiny in grain and used as a whole grain (Tadele and Hibistu, 2021). When it comes to producing *injera*, many people believe that *teff* grain is superior to other cereal grains (Dasa and Binh, 2020; Tadele and Hibistu, 2021). The Ethiopian flat and multi-eyed bread *injera*, with a sour taste that is best made using *teff* grain (Yigzaw *et al.*, 2001; Schneider and Anderson, 2010). According to Stewart and Getachew, (1962) *teff* flour, water and *ersho* (a liquid collected from previously fermented mixture) are combined to make *injera*, a thin, multi-eyed flat bread.

### **2.7.1. Fermentation process of *injera***

The making of *injera* dough requires two stages of fermentation. Depending on ambient temperatures, the first fermentation, may last from 24 to 72 hours (Mulaw and Tesfaye, 2017). After partially cleaning the fermentation container for backslopping, water is added to the milled and sieved *teff* flour. After adding *ersho* as an inoculum source, the mixture is allowed to ferment for 48 hours. The first 18 hours of fermentation is characterized by high dough rise and rapid gas generation. Next, a clear, yellowish liquid appears on the surface of the dough (Baumgarthuber, 2021).

At the end of the first stage of fermentation, this liquid layer is eliminated. After the liquid layer is removed, *absit* is made by mixing water with around 10% of the fermenting dough and boiling it for two to five minutes (Ashenafi, 2006; Gamboa and Ekris, 2008). *Absit* is a dough enhancer that is mixed in with the rest of the fermenting dough in the fermentation vat (Sahlin and BM Nair, 2012; Gashe, 1985). *Absit* makes ensuring that *injera* has the proper consistency and texture (Ashenafi, 2006; Gamboa and Ekris, 2008; It also speeds up the processes of dough-rising and gas formation (Gashe, 1985). The start of the second stage of fermentation is signaled by rapid fermentation process. The fermented dough, which has the right consistency of batter, is poured onto the hot oiled surface of the *metad*, which is a round smooth clay griddle, and baked for a few minutes to produce the large pancake-like *injera*.

### **2.7.2. Microorganisms involved in injera fermentation**

Fermented foods are linked with a unique group of microflora that enhance the nutritional quality of food such as proteins, vitamins, essential amino acids and fatty acids (Anal, 2019). *Teff injera*, a pancake-like acidic food is prepared from fermented *teff* (*Eragrostis teff*), flour. A complex group of microorganisms are involved in its fermentation. Fermentation is initiated by members of the Enterobacteriaceae. The dough pH is lowered to roughly 5.8 by their actions during the first 18 hr of fermentation. At this stage *Leuconostoc mesenteroides* and *Enterococcus faecalis* took over, and members of aerobic mesophilic bacteria which mainly consists of *Bacillus* spp (Gashe, 1985). This *Bacillus* spp. responsible to breakdown of starch into simple sugars which subsequently serves as substrate to initiate growth of organisms involved in fermentation (Adergoke and Babalol, 1988). As the pH is further reduced to about 4.7, *Pediococcus cerevisiae*, *Lactobacillus brevis*, *Lactobacillus plantarum* and *Lactobacillus fermentum* become the predominating flora and remain so until the fermentation is terminated at 72 hr. The dough acidic properties are caused by these lactic acid bacteria. Yeasts only appear in significant numbers at a later stage of the fermentation (Gashe, 1985). Yeasts responsible for *injera* fermentation are *Pichia fermentans*, *Pichia occidentalis*, *Candida humilis*, *Saccharomyces cerevisiae*, and *Kazachstania bulderi* species in fermented *injera* batter (Tadesse *et al.*, 2019).

### **2.7.3. Antinutritional factors of teff**

For minerals to be used for normal metabolic functions (bioavailable), they need to be absorbed through the small intestine (Fairweather-Tait, 2002). The bioavailability of minerals depends on subject/host and dietary factors (Hurrell and Egli, 2010). *Teff* are traditionally fermented into a variety of foods and beverages in order to improve organoleptic properties and, nutritional content, and digestibility (Gebremariam *et al.*, 2014).

Similar to other cereals, *teff* may contain considerable amount of antinutritional factors like, phytic acid, tannins and trypsin inhibitors (Knuckles, 1982). The mechanism by which phytate inhibits mineral absorption is based on the formation of insoluble phytate-mineral or peptide-mineralphytate complexes in the gastrointestinal tract (Weaver and Kannan 2002). Tannins might also reduce protein digestibility by inhibiting the digestive enzymes (Hewitt and Ford, 1982). High levels of trypsin inhibitor activity stimulate pancreatic juice secretion and cause pancreatic hypertrophy and growth (Liener, 1976). To reduce the levels of antinutritional factors in *teff*,

traditional processing methods such as fermentation, soaking, sprouting, and cooking can be employed. These methods help to break down or deactivate the antinutritional factors, making the nutrients in *teff* more bioavailable and easier to digest (Gebremariam *et al.*, 2014).

#### **2.7.4. Injera spoilage**

Microscopic spores drifting through the atmosphere give rise to mold. Some of these spores develop into mold when they land on wet food. According to Aidoo and Nout, (2010) ), food mold reproduces by generating compounds that cause the food's constituents to decompose and begin to decay. Filamentous molds are primarily responsible for two processes in fermented foods and alcoholic beverages: the synthesis of enzymes and the breakdown of anti-nutritive substances (Aidoo and Nout, 2010). Fungi are widely distributed in nature and their prevalence is one of the major causes of food spoilage (Marcet-Houben *et al.*, 2012). Therefore, where other microbes like bacteria cannot thrive, mold can grow on acidic goods like fruit juices and fruits (Lahlali *et al.*, 2005) and moderately moist meals like bread, *injera*, and baked goods (Abellana *et al.*, 1999).

The process of making *injera* involves numerous phases, such as preparing the grains and baking them every three days. The finished product is then kept in a traditional mosseb. All of these procedures are still done by hand using customs and local knowledge. *Injera* should not be stored at room temperature for longer than three days under normal conditions (the highlands of Ethiopia usually experience temperatures between 17 and 25°C). This is mostly because mold can cause spoilage. It's standard procedure to throw away moldy *injera*. Nonetheless, moldy *injera* is sun-dried and ready to eat during food shortages (Ashagrie and Abate, 2012). It degrades easily and loses quality due to its perishable nature. Furthermore, two main characteristics that make *injera* dangerous and unsatisfactory are mold growth and texture loss, that make *injera* unsafe and unacceptable for human consumption after storage at room temperature for approximately 3–4 days. Mold is a small, dusty place that spreads to a variety of foods and costs the economy millions of dollars each year (Bavaro *et al.*, 2017).

## **2.8. Ersho**

In order to make *teff injera* a starter (*Ersho*) saved from the previous fermented batter is utilized to start fresh batches of fermentation (Desiyeand Abegaz, 2013). Traditionally, to start new fermentation, a small portion of *ersh*, which has a complex group of microorganisms is used as a starter (Ashenafi, 1994). *Ersh*, *teff* flour, and water are carefully mixed by hand to make thin dough, which is then allowed to ferment. The liquid that separates on top is discarded and fresh water is added. The fermentation time, on the other hand, is determined by the environmental conditions (usually the room temperature/the ambient condition), the *ersh* composition, and the type of container utilized (Tewodros and Geremew, 2013).

*Ersh* has a pH below 4 and a titratable acidity of 4.46 percent, according to a research of its microbial flora and chemical properties (Ashenafi, 1994). The pH drops quickly when *ersh* is used (Yigzaw *et al.*, 2004). As a result, it does not promote the survival of various groups of microorganisms. *Ersh* is composed of minerals, carbohydrates, proteins, and amino acids. The general decrease in sugar levels in *ersh* with increased fermentation time is possibly due to the conversion of sugars to glycolytic end products such as lactic and acetic acids (Harrigan, 1998). ). Moreover, reductions in sugar and starch contents attributed to the activity of flour amylases during dough fermentation (Umeta and Faulks, 1989).

## **2.9. Traditional approaches of fermentation**

### **2.9.1. Spontaneous fermentation**

Food has been preserved through spontaneous fermentation, or processes carried out without the aid of a starter inoculum, for centuries and have been explained by trial and error, most likely throughout thousands of years (Akpi, *et al.*, 2020). Spontaneous fermentation is typically the result of competitive activities of a variety of contaminating microorganisms (Holzapfel, 1997) (Adegoke, *et al.*, 1994). The existence of microorganisms that are native to the raw materials and fermenting container is the foundation for spontaneous fermentations.

However, in order to serve as starter cultures, preventing the growth of pathogenic bacteria and undesired degradation, these microorganisms must be able to multiply rapidly and at large quantities (Holzapfel, 2002; Montel *et al.*, 2014; Franciosa *et al.*, 2018). The microorganisms that cause spontaneous fermentation are typically a consortium of bacteria, fungi and/or yeasts, which provides the typical sensory characteristics of the products obtained (Smid and Kleerebezem, 2014).

The foundation of traditional fermented foods and drinks is spontaneous fermentation and are generally difficult to standardize products. During the fermentation process, indigenous microorganisms are generally a consortia of microorganisms rather than pure cultures (Smid and Lacroix, 2013). Yeasts follow bacteria in the early stages of fermentation processes because of their relatively rapid growth rate on substrates high in fermentable sugars (Holzapfel, 2002). A spontaneous fermentation process has a high failure rate and requires a considerable amount of time to start (24–48 hours) (Holzapfel, 2002). During this early stage, the contamination of raw materials, utensils, and the environment by microbes grows gradually during this early stage and competes for nutrients, which is connected to the lag phase of microbial development (Akpi, *et al.*, 2020) This phase can be shortened by using either back-slopping or specific starter cultures. Beneficial attributes of the substrate, consumer expectations and technical requirements dictate to a large extent the nature of the starter culture to be used, i.e. single-strain versus mixed-strain culture (Holzapfel, 2002).

### **2.9.2. Back-slopping**

Spontaneous fermentation formed the basis for the earliest production of fermented foods, because the naturally occurring bacteria in the raw material developed. The quality of the end product was affected by the microbial load and spectrum of the raw material. Spontaneous fermentation was optimized through back slopping. Using the back-slopping method, a tiny amount of fermented products is mixed with fresh ingredients, and the mixture is then left to ferment at room temperature. After a few rounds, a stable microbial community finally forms (Schoustra *et al.*, 2013).

Back-slopping fermentation produces stable microflora, it guarantees the safety of the finished product and can improve food quality (Li and Gänzle, 2020). The best-adapted strains become dominant when there is backslopping. This technology is still used for production of foods and beverages where the ecology and specific knowledge about microbial population and role are not clearly known. This is another economical and reliable method produce fermented foods (Campbell-Platt, 1994). Back slopping is still used, for example, to make sourdough and sauerkraut, and especially for products whose microbial ecology and the precise function of successions in the microbial population are unclear (Harris, 1998).

In numerous traditional processes, material from a previous successful batch is often incorporated in many classical procedures. Back-slopping is a technique that shortens the fermentation period and lowers the possibility of a failed fermentation (Holzapfel, 2002). Therefore, back-slopping causes the strains that are best adapted to the fermentation process to dominate. It represents a way, be it unconsciously, of using a selected starter culture to shorten the fermentation process and to lower the chance of fermentation failure (Holzapfel, 2002).

## **2.10. Starter cultures**

Starter cultures are defined as preparations having a high number of cells, either of a single species or a mixture of two or more microorganisms, in order to benefit from the chemicals or products acquired from their metabolism or enzymatic activity (Fox *et al.*, 2017). The use of starter culture during fermentation can increase the safety of fermented food by improving the process, allowing control over the initial stage of fermentation and the predictability of derivative products, as well as lowering organoleptic fluctuations and microbiological instability. (Kimaryo *et al.*, 2000; Holzapfel, 2002).

The fermentation process involves two main factors: the first factor is the type of microorganisms used and the second factor is the substrate. Microorganisms are responsible to initiate the fermentation process of the food, which involves the chemical transformation of primary compounds into new secondary metabolites, that generate organoleptic changes in the product, and thereby, extending the shelf life of the product (Tamang *et al.*, 2016) . These microorganisms can be yeasts, fungi or bacteria (Bourdichon *et al.*, 2012), depending on the substrate to degrade, since the mechanism of the fermentation process varies according to the substrate. The substrates can be

meat, fish, dairy, vegetables, cereals, and fruits, including food wastes such as fruit peels (Marco *et al.*, 2017). starter cultures are adapted to the substrates, they allow us control of the fermentation process to obtain predictable results (Franciosa *et al.*, 2018).

Microorganisms are responsible to starting the fermentation process of the food, which entails the chemical transformation of primary compounds into new secondary metabolites, that produce organoleptic changes in the final product and increase its shelf life (Tamang *et al.*, 2016). Since they are well suited to the environmental conditions of food and have the ability to regulate food spoilage and pathogenic microbiota, the most promising microorganisms selected as starter culture are those that are isolated from the native microbiota of traditional products (Pereira *et al.*, 2020; Laranjo *et al.*, 2019).

To select a microorganism(s) as a starter or to develop starter culture, it is essential to do a thorough investigation into the metabolism and activities (Díez and Patarata, 2013). Selected strains may specifically contribute to biological enrichment through the production of vitamins and critical amino acids, as well as improve the general benefits of spontaneous fermentation, such as improved protein digestibility and micronutrient bioavailability (Holzapfel, 2002). Molds, yeast, and bacteria are the microorganisms that are employed as starter cultures (Ray and Joshi, 2014).

### **2.10.1. Lactic Acid Bacteria**

According to Meim and Sulieman (2018), starter cultures are a preparation used to help the fermentation process get started while making different foods and fermented beverages. The Lactobacillaceae, which can make lactic acid from carbohydrates, are the most significant bacteria in food fermentation (Leroy and De Vuyst, 2004). While some LAB are heterofermentative and additionally create ethanol and carbon dioxide, others are homofermentative and only produce lactic acid as the end result of the fermentation of glucose (Blandino *et al.*, 2003). The LAB strains antibacterial activity might aid in the fight against microbial contamination (Holzapfel *et al.*, 1995; Lucke, 2000). Natural antimicrobials produced by LAB include carbon dioxide, hydrogen peroxide, diacetyl, ethanol, organic acids (lactic, acetic, formic, phenyllactic, and caproic acid), and bacteriocins (such as nisin, reuterin, and reutericyclin). For example, acetic acid keeps sourdough from spoiling due to mold and adds to its aroma (Messens and De Vuyst, 2002).

According to Sengun and Karabiyikli (2011), the microorganisms most frequently discovered in fermented foods are lactic acid bacteria (LAB). Their physiological traits, including as substrate consumption, metabolic capabilities, and probiotic qualities, are linked to their critical importance. Food quality can be raised during fermented food processing by the production of monosaccharides, lactic acid, and other byproducts from the breakdown of polysaccharides by lactic acid bacteria (Wang *et al.*, 2021). In fermented food production, lactic acid bacteria are widely used and make up the majority of the volume and value of commercial starter cultures. In food fermentation, the culture's main job is to convert sugar into desired byproducts such CO<sub>2</sub>, acetic acid, lactic acid, or alcohol (Moneim and Sulieman, 2017).

LAB have been extensively used in food fermentation, including the production of milk products, and its proteolytic activity is very important in producing flavor compounds of end product (Moulay *et al.*, 2013). The activity of LAB has been essential to the fermentation process of milk, since it transforms raw milk into fermented milk products. The most significant characteristics of LAB are their capacity to produce flavor and texture, acidify milk, and change milk protein through their proteolytic activities (Mäyrä and Bigret, 2004). Lactobacilli are used as starters when making mozzarella cheese and yoghurt. Additionally, they are also used as starter subordinates to promote faster ripening of Cheddar and comparable cheeses, to decrease the rate of intensity of bitterness and as probiotics in yoghurt type products. *Lactobacillus delbrueckii* subsp. *bulgaricus* is widely used along with *Streptococcus thermophilus* as a starter in yoghurt production (Moneim and Sulieman, 2017).

### **2.10.2. Aerobic mesophilic bacteria**

Mesophilic aerobic bacteria are helpful in assessing the hygienic condition of food and can be utilized as markers for determining the shelf life and sanitary quality of food (Ray, 2004; James *et al.*, 2005). It is important to exercise caution when using mesophilic aerobic bacteria as indicators because certain meals, like fermented foods, naturally contain large amounts of bacteria (Canberra, 2017). Their optimal growth temperature is 32°C, however they can grow in a temperature range of 20 to 45°C (Jay *et al.*, 2005). The bacteria that are frequently employed to confirm the microbiological quality of milk and dairy products are useful because they reveal important details about the hygienic and sanitary conditions of milking, storing, and processing, as well as indicating the potential existence of harmful microorganisms (ICMSF, 1988). AMB and Enterobacteriaceae

are two types of microbes frequently found in industrial environments. With a few exceptions, they don't pose a risk to human health until they are present in certain amounts in food (Putnik *et al.*, 2017).

### **2.10.3. Yeast**

In olden times people unknowingly used yeasts for producing fermented foods and beverages; the knowledge of these microorganisms ability to alter carbohydrates into ethanol and carbon dioxide (CO<sub>2</sub>) is demonstrated in the 1860s by Louis Pasteur (Buzzini *et al.*, 2017). Yeasts are eukaryotic, unicellular organisms found in a wide range of traditional fermented foods manufactured from plant and animal base materials (Tamang and Fleet, 2009). Yeasts play a significant role in food fermentation through enzyme production that favours desired biochemical reactions, such as production of flavour, alcohol and aroma (Aidoo *et al.*, 2006). According to some theories, the first examples of organisms that humankind were those that belonged to the *Saccharomyces sensu stricto* complex, which includes *Saccharomyces cerevisiae*, also known as "baker's yeast" (Sicard and Legras, 2011). Additionally, genera of *Candida spp.*, *Endomycopsis spp.*, *Hansenula spp.*, *Pichia spp.*, *Rhodotorula spp.*, *Saccharomycopsis spp.* and *Torulopsis spp.* is getting attention for their promising utilization as starter cultures in both food and non-food (industrial) applications (Buzzini and Vaughan, 2006).

Yeast transforms sugar into carbon dioxide and alcohol during dough fermentation, which affects the baked goods texture. The most common type of yeast used as a leavening agent in baked goods is *Saccharomyces cerevisiae*, sometimes known as baker's yeast (Newberry *et al.*, 2002). *Saccharomyces cerevisiae* is the yeast species that is most commonly recovered, followed by *Kazachstania humilis* and *Wickerhamomyces anomalus* (De Vuyst *et al.*, 2016). *Lactobacillus sanfranciscensis* is the predominant species among LAB (Gänzle and Ripari, 2016).

### **2.10.4. Molds**

Molds usually form long, highly branched cells, and easily grow on moist, solid nutrient surface. The growth of molds in the form of pellets can be an alternative in industrial fermentation processes. Two forms of fungi that are frequently present in fermented foods are molds and yeasts, which are necessary for the creation of flavoring compounds and the synthesis of functional ingredients. Despite being an ancient bioprocess, traditional fungal fermentation used to produce

foods and beverages is still in use worldwide (Yang *et al.*, 2022). Many fungal species grow on food products, often contributing to their spoilage, as in fruits, vegetables, and other fresh or prepared products. Nonetheless, the existence of fungi on certain items often has no negative consequences and may even contribute to the ripening and development of important organoleptic characteristics typical of a particular food (Grazia *et al.*, 1986; Huerta *et al.*, 1987).

The main role of molds in fermented food is to produce a variety of enzymes (Tamang, 2010). For example, protease, amylase, glutamidase, pectinase, hemicellulase and cellulase can use starch, oligosaccharide and monosaccharide as carbon source, and protein, amino acid and urea as nitrogen source. The two primary roles of filamentous molds in fermented foods and alcoholic beverages are the production of enzymes and the degradation of antinutritive compounds (Aidoo and Nout, 2010). Many moulds have capacity to produce enzymes of commercial importance such as pectinase by *Aspergillus niger* (Joshi *et al.*, 2006). Similar to this, molds are successfully used in the synthesis of fat and single-cell proteins, as well as in the synthesis of hormones, vitamins, ethyl alcohol, and antibiotics (Uraz, 2014).

Some of the fungi that are most commonly used as starters are *Penicillium nalgiovense* and *Penicillium chrysogenum* for meat products, *Penicillium roqueforti* for Roquefort cheese, and *Penicillium camemberti* for Camembert cheese (Laich *et al.*, 2002). Furthermore, mold (*Penicillium notatum*-*chrysogenum* group) produces a number of antibiotic groups, including penicillin (Shuler, 2002). Moreover, molds have the ability to biosynthesize of toxic secondary metabolites, commonly known as mycotoxins. Some of them have proven to have a carcinogenic (fumonisin B1, aflatoxin B1, ochratoxin A), mutagenic (aflatoxins, fumonisins, ochratoxin A, toxin T-2), teratogenic (patulin, aflatoxin B1, ochratoxin A), estrogenic (zearalenone), nephrotoxic and hepatotoxic (aflatoxins, patulin) effect. Different types of mold have different optimum temperatures for growth; some can even thrive in refrigerators. Their secondary metabolism is varied and yields a wide range of poisons and carcinogens. Certain decaying molds are poisonous, although not all of them are ((Pitt and Hocking, 1977).

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aflatoxin B1, ochratoxin A), estrogenic (zearalenone), nephrotoxic, and hepatotoxic (aflatoxins, patulin) effects. Different types of mold have different optimum temperatures for growth; some can even thrive in refrigerators. Their secondary metabolism is varied and yields a wide range of toxins and carcinogens. Certain decaying molds are poisonous, although not all of them are (Pitt and Hocking, 1977).

### 3. Materials and Methods

#### 3.1. Sample collection and description of the study sites

A total of ninety-one (91) samples of *teff ersho* (fermented dough left from previous fermentation for back slopping) were collected from households in the highlands of Debrebirhan, Fiche, Chacha, Sebata, Teji, and Holeta sampling sites and the lowlands of Adama, Bishoftu, and Dukam. The samples ranged in age from one to ninety-six hrs. Using sterile falcon tubes, *Erscho* samples were obtained from households and stored in a refrigerator till processing. Samples of 15 Adama 15 Debrebirhan, 12 Bishoftu, 12 Dukam, 4 Fiche, 4 Chacha, 4 Sebata, 15 Teji and 15 Holeta were collected in total.



Figure 2. Sample collection events

##### 3.1.1. Experimental location

The experiments were done at the Microbial Biotechnology Laboratory of the Holeta National Agricultural Biotechnology Research Center, which is located 29 kilometers west of Addis Ababa. Then, Molecular identification was carried out at the MRC-Et Advanced laboratory.

##### 3.1.2. Experimental design

Experimental work was done by using completely randomized design (CRD) method. CRD was used for the experimental design for the microbial analysis of the experiment in triplicate.

### 3.2. Determination of pH

The pH of *ersho* was measured by using a pH meter (pHS-3C, China). The pH meter is calibrated against standard buffers before measuring the pH of sample. pH meters are provided with operating instructions, which should be followed. Rinse the electrode with water and wipe dry using a tissue in between buffer and sample pH measurements

### 3.3. Titratable Acidity

To measure titratable acidity 10 ml of *ersho* was taken and 3-5 drops of phenolphthalein indicator were added. Then an alkaline solution of 0.1N NaOH was added until the neutralization point is reached (turn to pink). When a phenolphthalein indicator, was turned from colorless to pink. The total acidity was calculated as the percent lactic acid (Fabro *et al.*, 2006). Calculation of the % titratable acidity was as follows:

$$\% \text{ lactic acid} = \frac{\text{ml of NaOH} \times 0.1\text{N NaOH} \times 0.0908 \times 100}{\text{ml of sample}}$$

Where ml = ml 0.1N NaOH

N = Normality of 0.1N NaOH

0.0908 = Molecular weight of Lactate

### 3.4. Isolation and identification of microorganisms

For isolation of 1 ml *ersho* samples were transferred into test tubes of 9 ml sterile saline water and homogenized by using vortex mixer. Then after, serial dilutions were made to obtain countable numbers of colonies on their own agar plates (MRS, Nutrient agar, PDA for lactic acid bacteria, aerobic mesophilic bacteria and PDA respectively) (Yousef and Carlstrom, 2003). Aliquots of 0.1 ml from appropriate dilutions were spread plated on their respective agar plates. The samples were incubated at their own optimum temperature (35°C for LAB & AMB and 25°C for yeast & mold). Representative colonies from enumerated plates were purified on their own media and purified isolates were transferred to slant cultures and preserved at 4°C for further study.

#### **3.4.1. Aerobic mesophilic bacteria**

Aerobic mesophilic bacteria were enumerated by spread plating 1ml of each of the sample was transferred to 9 ml of sterile distilled water to be successively diluted to  $10^{-1}$  up to  $10^{-6}$ . Aliquots of 0.1 ml from dilutions  $10^{-4}$  and  $10^{-5}$  were spread onto duplicate, Nutrient Agar (Oxoid Ltd, Basingstoke, Hampshire, UK) plates. Colonies were counted after incubation at  $35^{\circ}\text{C}$  for 2 days. The traits of each species were identified using the guidelines provided in Bergey's Manual (Claus and Berkeley, 1986). Then after, To purify the isolates, the selected colonies were transferred to nutrient broth and cultivated on nutrient agar. A total of 82 representative colonies of AMB were selected on the basis of their morphological trait.

#### **3.4.2. Lactic acid bacteria**

Lactic acid bacteria (LAB) were serially diluted and spread-plated to count onto MRS agar. Aliquots of 1ml from dilutions  $10^{-4}$  and  $10^{-5}$  were spread onto duplicate, de Man, Rogosa, and Sharpe (MRS, HIMEDIA, (M091 Darmstadt, Germany) agar plates. After three days of incubation at  $35^{\circ}\text{C}$  in an anaerobic culture jar (polycarbonate, India), colonies were counted. Lactic acid bacteria were identified as yellow/white glossy colonies on MRS agar (Hoque *et al.*, 2010). Representative colonies were picked by considering the colony characteristics (i.e., shape, colour and size) and transferred to MRS broth. Then after, streaked on fresh MRS agar plates to have pure single colonies. Purified LAB colonies were preserved for further analysis. A total of 150 representative colonies of LAB were selected.

#### **3.4.3. Yeasts and Molds**

A 1ml of each of the sample was transferred to nine ml of sterile distilled water to be successively diluted to  $10^{-1}$  up to  $10^{-6}$ . Aliquots of 0.1 ml from dilutions  $10^{-4}$  and  $10^{-5}$  were spread onto duplicate Potato Dextrose Agar (PDA) (Sigma-Aldrich, Darmstadt, Germany) plates. Yeast colonies were counted after incubating the plates at  $28^{\circ}\text{C}$  for 3 to 5 days. Molds were incubated for 5 to 7 days. After incubation, Single-spore isolations were performed in accordance with Crous *et al.*, (2009). A total of 161 yeast and 55 mold typical colonies were chosen based on the morphological characteristics of the colony, such as its size, shape, and color.

### **3.4. Preservation of the pure isolate**

Pure isolates were cultured on their own broth (MRS, Nutrient agar, PDA for lactic acid bacteria, aerobic mesophilic bacteria and PDA respectively). For short term preservation fresh culture of yeast and mold were purified by sub culturing on PDA medium by streaking and incubated at 28<sup>0</sup>C 3 to 5 days . The pure culture was kept on YPD broth and stored at 4<sup>0</sup>C for further study. Lactic acid bacteria (LAB) were purified by sub culturing on MRS agar plate by streaking and incubated at 35<sup>0</sup>C for 3 days. The pure culture was kept on MRS broth and stored at 4<sup>0</sup>C for further study. Aerobic mesophilic bacteria cultured by streaking on nutrient agar plate for purification. The pure culture was kept on nutrient broth and incubated at 28<sup>0</sup>C for 2 days then, stored at 4<sup>0</sup>C. For long term preservation fresh culture of each isolates were refreshed on (MRS, Nutrient agar, PDA for lactic acid bacteria, aerobic mesophilic bacteria and PDA respectively) and inoculated to broth containing glycerol (10% v/v) and finally stored in freezer (-20<sup>0</sup>C and -80<sup>0</sup>C).

### **3.5. Screening of microorganisms**

#### **3.5.1. Preliminary screening**

Lactic acid bacteria, AMB, yeast and mold were refreshed on MRS broth, Nutrient Agar and PDA plates, respectively. To screen for acid production from sugar broth medium containing (peptone 10 g, beef extract 1 g, NaCl 5 g, phenol red 0.018 g and sugar 10 g per liter) was used to select best isolates from purified (Aerobic Mesophilic Bacteria (AMB), Lactic acid bacteria (LAB) and yeast. Separate test tubes were filled with 9 ml of sugar broth. Fresh culture 1ml of the purified isolates were inoculated into the sterilized sugar broth. To screen for acid production among the purified LAB and AMB isolates in sugar broth phenol red as an indicator was utilized. Change of color from red to yellow signifies that the organisms utilized sugar to produce acidic products. Isolates that changed the red to yellow color were selected.

Durham tubes were used for testing of yeasts for gas production. They were inserted upside down inside test tubes. After sterilization, The sugar broth medium was kept at 28<sup>0</sup>C to cultivate the yeast isolates. The inverted durham tubes were trap released gases as an air bubble (Reiner, 2012). The color change of the media from reddish-orange to yellow indicates the occurrence of fermentation and the formation of an air bubble in durham's tube indicates the release of gas.

The capacity of molds to break down Carboxyl Methyl Cellulose (CMC) agar medium agar medium was evaluated. The following components were present in the medium: 0.05 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.005 g CaCl<sub>2</sub>, 0.005 g NaNO<sub>3</sub>, 0.009 g FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.002 g ZnSO<sub>4</sub>, 0.012 g MnSO<sub>4</sub>, 0.23 g KCl, 0.23 g KH<sub>2</sub>PO<sub>4</sub>, 2 g peptone, 19 g Agar (Kusumawati *et al.*, 2010). Following five days of incubation at 28°C, each plate was stained with 0.4% Congo red for ten minutes, and then it was destained with 1 M NaCl. After measuring the hydrolysis zones on the plate media, isolates that had a greater clear zone surrounding the mold colony on the agar medium were chosen and put through a further screening process.

### **3.5.2. Secondary screening**

#### **3.5.2.1. Sugar utilization test**

Lactic acid bacteria, AMB, yeast and mold were refreshed on MRS broth, Nutrient Agar and PDA respectively. The sugars used for fermentation by LAB, AMB, mold and yeasts as a test used were xylose, fructose, sucrose, raffinose, maltose, and starch. The carbohydrate utilization test was performed using broth (peptone: 10 g; NaCl: 5 g; beef extract: 1 gm; phenol red: 0.018 g; distilled water: 1000 ml; carbohydrate: 10 g) (Reiner, 2012). The utilization of the sugars of selected isolates (a total of 40, 49, 56 and 24 of LAB, AMB, yeast and mold, respectively) were determined by applying 0.1 ml of culture on 1 ml of sugar broth deep well plates. The control group consisted of sugars without an inoculum in the first row of the well. The inoculated plates containing the isolates from each group were incubated at 35 °C for LAB and AMB and 28 °C for yeast and mold. The microplate reader (SkanIt Software 5.0 for Microplate Readers RE, ver. 5.0.0.42, China) was used to measure the growth of each isolate at 630 nm.

#### **3.5.3. Temperature tolerance test**

A total of 40, 49, 56, and 24 isolates of LAB, AMB, yeast, and mold, respectively, were inoculated into their broth medium for the temperature tolerance test. 0.1 ml of refreshed cultures were transferred into the deep well plate which contain 1 ml MRS broth for LAB and nutrient broth for AMB and incubated for 24hrs. The deep well plate was incubated for 48 hours with 1 ml YPD broth and 0.1ml culture applied for both yeast and mold. After inoculation, they were incubated at the following temperatures: 15°C, 25°C, 45°C and 55°C for LAB and AMB, and 15°C, 35°C 45°C

and 55<sup>0</sup>C for yeast and mold. Absorbance was read at 630 nm in a microplate reader (SkanIt Software 5.0 for Microplate Readers RE, ver. 5.0.0.42, China) and their growth was recorded.

#### **3.5.4. Acid stress test**

Lactic acid bacteria, AMB, yeast and mold were subjected to acid stress test on their respective broth (MRS, Nutrient Agar and PDA respectively) by adjusting the pH of the tubes to 2, 3 and 4 with 1 N of HCl. 0.1 ml of refreshed cultures were added into 1 ml adjusted pH broth on deep well plate. A total of 40, 49, 56 and 24 isolates of LAB, AMB, yeast and mold, respectively were inoculated separately into each well with triplicate and incubated at 35 <sup>0</sup>C for LAB and AMB for 24hrs and 28 <sup>0</sup>C yeast and mold incubated for 48 hrs. Finally, the impact of pH on the growth of selected isolate was recorded at 630 nm.

#### **3.5.5. Hydrogen sulfide production test**

The fresh culture of LAB, AMB, yeast and mold isolates, were streaked on agar slant prepared with triple sugar iron agar (TSIA). Triple Sugar Iron Agar is used to identify specific isolates based on the fermentation of glucose, lactose, and sucrose in addition to beef extract and peptones (Lehman, 2006). They were incubated for 3-5 days at 28 <sup>0</sup>C (yeast and mold) and 35 <sup>0</sup>C (LAB and AMB). Isolates that produce hydrogen sulfide (H<sub>2</sub>S) from the fermentation were identified and rejected.

#### **3.6. Viability of isolate**

Cell viability was estimated through plate count (Hedges, 2002 and Gaglio *et al.*, 2015), with some modification. The viability test for selected isolates of lactic acid bacteria, yeast, mold and aerobic mesophilic bacteria was checked by spread plate on their respective agar media under stress conditions such as temperature (25, 35 and 45 <sup>0</sup>C ) and pH (2 and 3). Viability was assessed at 24 and 48 hrs. The analysis was performed in duplicate and resulting colonies were counted. In addition to viability the isolates that performed the best were determined by comparing their means using the sugar utilization test, temperature stress test and pH stress test.

### **3.7. Compatibility test**

The compatibility test was conducted by cross-streaking the six selected isolates for starter culture formulation. The isolates include 2 lactic acid bacteria, 2 yeast, 1 aerobic mesophilic bacteria and 1 mold. The treatments were done by streaking the isolates perpendicularly on agar medium according to (Sci, 2008). After incubation, based on the existence or lack of a zone of inhibition in the streaked region of two isolates, the compatibility of the isolates were checked.

### **3.8. Starter Culture Formulation**

The starter culture was prepared by using LAB, AMB, yeast and mold that were determined the best during the screening process. The formulations were designed with for treatment groups having 59 combinations. Before use, the selected LAB and AMB isolates were separately refreshed in their respective broth media (MRS and Nutrient Broth) and incubated at 35<sup>0</sup>C for 2 days. After being refreshed in YPD broth, the yeast and mold were cultured for 3 days at 28<sup>0</sup>C. The refreshed cultures of isolate were centrifuged at 5000 rpm for 5 minutes in order to prepare the formulation of a defined starter culture. After that, the pellet was then washed two times with sterile distilled water to get rid of the residual media. The pellet was dissolved in sterile distilled water and standardized to 0.5 Log<sub>10</sub> CFU/ ml<sup>-1</sup>. The 1%, 2%, 3% and 4% of standardized microbes were inoculated in 50 g *teff* flour mixed with 100 ml sterilized distilled water. Ultimately, the best % of microbes for fermentation was selected and employed for formulation. Sterile and non-sterilized flour were used for the combination. In order to determine how much water was added to the *teff*.

#### **3.8.1. Preparation of fermented dough and baking of *injera***

A 50 g of *teff* flour was mixed with 4 % of selected isolates (formulation), 100 ml of water and the dough was kneaded by sterilized spatula for each formulation /treatment (Mihrete and Bultosa, 2017). The dough was kept by covering the lid at 25<sup>0</sup>C until the fermentation ended. Maximum dough rise and noticeably vigorous gas evolution were noted during the 13hrs fermentation. Yellowish liquid on dough surface were observed at the end of first fermentation (25hrs). After that, the liquid layer was eliminated. Ten percent of the fermented dough was removed in order to get the absit ready for baking the *injera*. *Absit* was boiled, cooled and mixed back to the fermenting dough. *Injera* was baked on '*metad*' at the end of second fermentation (Mihrete and Bultosa, 2017). At the end of the fermentation process, the pH of the dough was measured using pH meter.

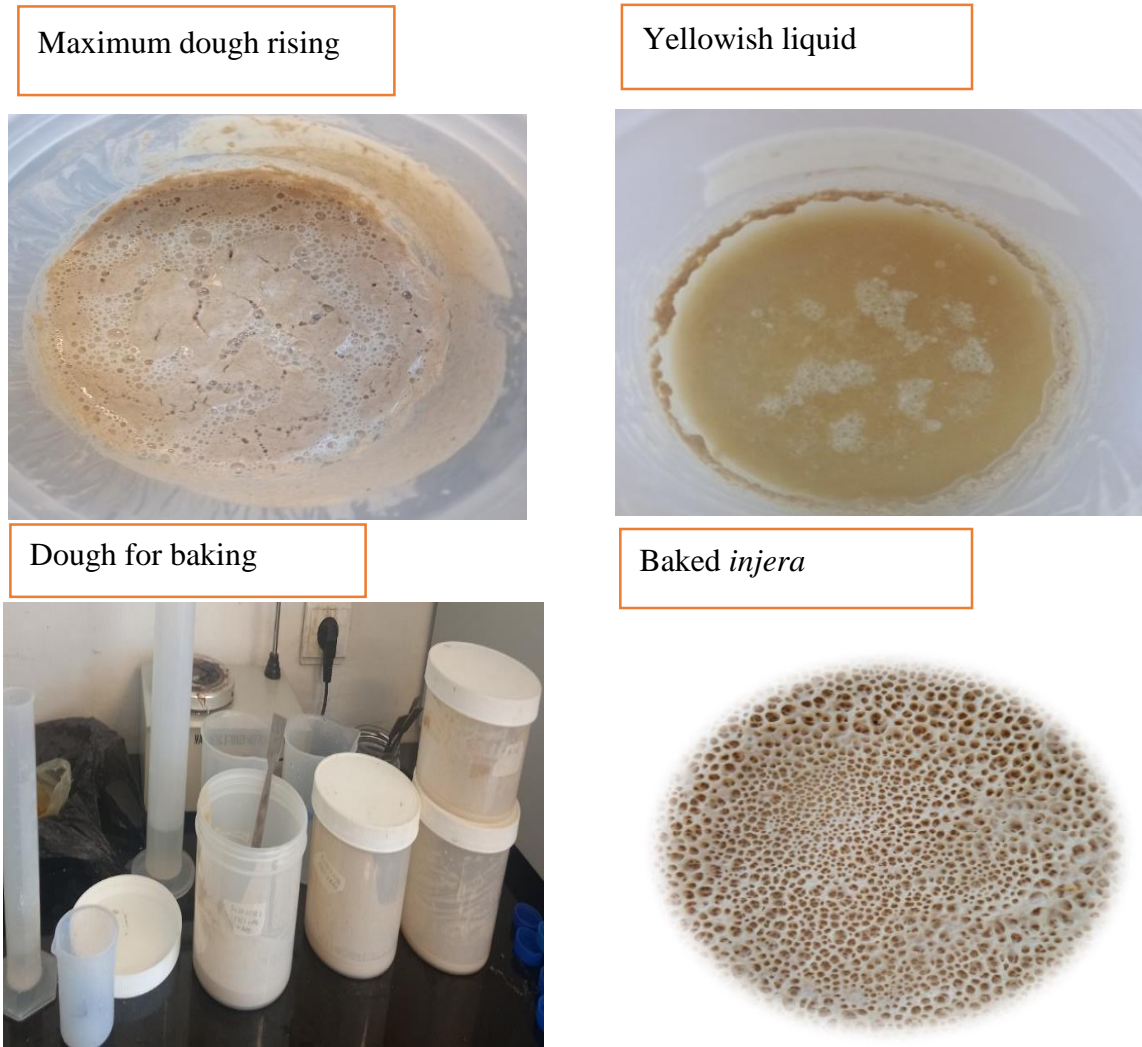


Figure 3. Dough preparation and baking of *injera*

**3.8.2. Sensory evaluation**

The baked *injera* prepared from the various combination of microbes were analyzed for sensory characteristics by a panel of 10 judges using 5-point of hedonic scale. The panelists composed of male and majority were female (4 male and 6 women) who regularly consume *injera* as their staple food, was selected following the criterion described by (Stone and Sidel, 2004). Six *injera* quality descriptors were used for evaluation: taste, texture, eye uniformity, odour, backside appearance and overall acceptability was evaluated (appendix 5). The five-point hedonic scale, which ranges

from 1 (dislike very much) to 5 (like very much), was used to evaluating degree of disliking and liking. Water was given to the panelists so they could wash their mouths after tasting each sample.



Figure 4. Sensory evaluation by panelists

### **3.9. Molecular Characterization**

#### **3.9.1. Genomic DNA Extraction**

Genomic DNA was extracted from bacterial pure cultures using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, and USA), according to the manufacturer's instructions. The primer pair rD1 and fD1 were used to generate a 1500bp PCR product (Weisburg *et al.*, 1991). The portions of intergenic region between the 16S and 23S rRNA was amplified using FGPS1490 and FGPS132 primers (Barry *et al.*, 1991). The primers of internal transcribed spacers (ITS1, ITS2, and ITS4) were utilized to extract yeast and mold isolates (Evelyn, 2009). The genomic DNA of isolates was extracted according the protocol of (Harju *et al.*, 2004).

#### **3.9.2. Amplification of 16S rDNA of the two Bacterial groups (LAB and AMB)**

The PCR amplifications were performed with both primers (Fd1 (5'AGAGTTTGATCCTGGCTCAG 3') and Rd1 (5'AAGGAGGTGATCCAGCC 3')). The PCR condition was a 95 degree initial denaturation for 5 minutes followed by 35 cycles of denaturation at 95 degrees for 30 seconds, annealing at 55 degrees for 1 minute and extension at 72 degrees for 30 seconds with a final extension 5-minute extension at 72 degrees. After running the PCR, the

amplicons of LAB and AMB were separated by gel electrophoresis using 3% agarose gel and 1 µL loading dye with 5 µL PCR products. The same primers were then used to sequence the PCR product on the ABI 3730XL genetic analyzer.

### **3.9.3. Amplification of ITS DNA of yeast and mold isolates**

To achieve a final reaction volume of 50 µl, PCR amplification reaction mixtures contained 3 µl of the extracted DNA, 1 µl of both primers 25 µl of master mix and 20 µl dH<sub>2</sub>O. The PCR cycle conditions include an initial denaturation step at 95°C for 1min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 1min, and primer extension at 72°C for 1min with a final extension at 72°C for 5min (Evelyn, 2009). PCR product was checked by gel electrophoresis using a 3% agarose gel and 2µl loading dye with 5µl PCR products .

### **3.9.4. DNA purification and sequencing**

All obtained PCR amplification products were purified using Amersham Biosciences and gel band purification kit, the results were sequenced were sequenced

### **3.9.5. Phylogenetic analysis**

The complete sequences were blasted at NCBI. Final sequences were then aligned using CLUSTAL (version: 1.2.4) (Tompson, *et al.*, 1994) for each of the sequences. The sequences of bacterial and fungal isolates of this study were then compared to those in GenBank (National Centre for Biotechnology Information; <http://www.ncbi.nih.gov/>) using the Basic Local Alignment Search Tool (Altschul *et al.*, 1990) for nucleotide sequences (blastn). Evolutionary analyses were conducted using the neighbor-joining algorithm by MEGA11 (Tamura and Nei, 1993).

### **3.10. Statistical analysis**

Data obtained from (OD) was analyzed using the R 4.2.0, Analysis of variance (ANOVA) and means comparison via the tukey test with a significance level of 0.05. The data collected on sensory evaluation was analyzed using SPSS version 20 and sigmaplot 14.0. The phylogenetic tree construction was accomplished by MEGA11 software.

## **4. Results**

### **4.1 PH and titratable acidity of *ersho***

A total of 91 *ersho* samples were collected from various households of different cities and towns around Addis Ababa. Their pH and titratable acidity values were scored. The *ersho* samples that were collected from the different households had pH values around 4.0. The titratable acidity ranged between 4.1% and 5.2% for all samples and showed significant variation within households.

### **4.2. Isolation of different groups of microorganisms**

Different groups of microorganism were isolated from *ersho* samples using appropriate medium for each group. Figure 5 exhibited different groups of organisms such as AMB on nutrient agar (Figure 5C), LAB on MRS Agar (Figure 5B), yeasts on PDA (non-hairy and smooth colonies, Figure 5A), and molds on the same PDA (hairy unlike yeasts, Figure 5D). The colonies recovered from the respective growth media were identified as lactic acid bacteria (LAB), Aerobic mesophilic bacteria (AMB), yeast and mold. Out of 91 samples collected and processed a total of 150 lactic acid bacteria (LAB), 82 Aerobic mesophilic bacteria (AMB), 161 yeast and 55 mold isolates were isolated and purified.

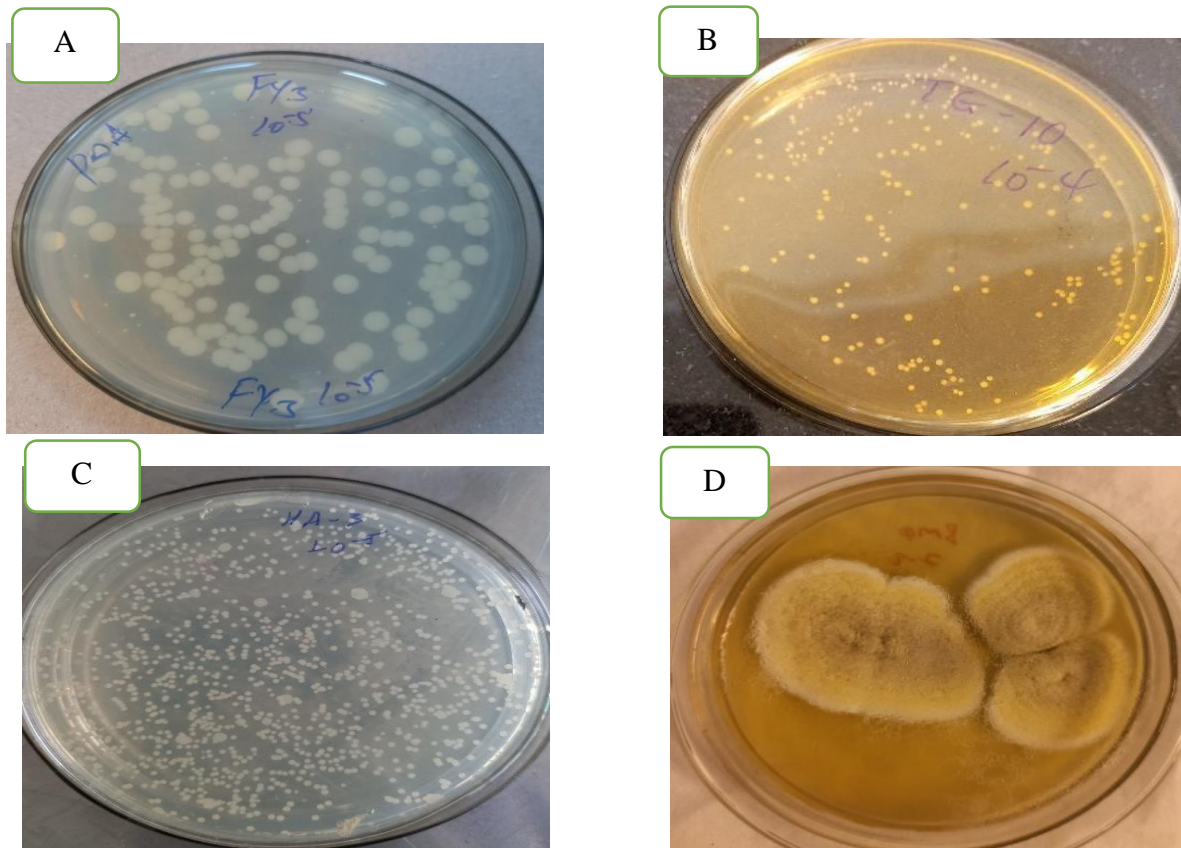


Figure 5. Different groups of microorganism isolated from *teff ersho*

A=Yeast B=Lactic acid bacteria C=Aerobic mesophilic bacteria D=Mold

### 4.3. Screening methods

#### 4.3.1. Preliminary test result

Yellow indicates that the microbes utilize sugar to produce acidic products, while the initial red test tube served as control (Figure 6A). Out of the tested isolates, 40 lactic acid bacteria and 49 AMB showed positive results for glucose utilization. For yeast isolates, a total of 56 were selected based on their glucose utilization result and fast gas producers were selected. In the case of mold isolates, based on the formation of a clear zone, 24 mold isolates were selected for further investigation (Figure 6B).

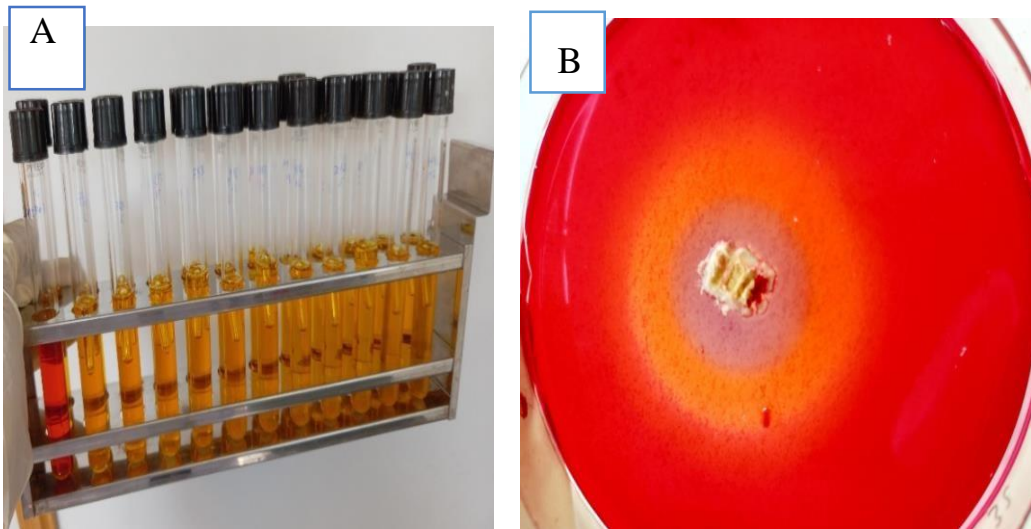


Figure 6. Preliminary test result for LAB, AMB, yeast and mold

A= sugar utilization , first test tube (red color) indicates control, yellow color test tubes indicates acid production B= Different groups of microorganism

#### 4.3.2. Sugar utilization result

Table 1 displays that all isolates of lactic acid bacteria (40), AMB (49), yeast (56), and mold (24) were able to ferment sucrose, maltose, fructose, xylose, raffinose and starch. The results indicate that all the isolates were able to ferment sugars to different extent (Table 1).

Table 1. Sugar utilization table with six sugar types for LAB, AMB, yeast and mold

<b>Types of microbes</b>	<b>Sugars</b>	<b>Mean±sd</b>	<b>f- value</b>	<b>p – value</b>
AMB	Fructose	0.37±0.1	6.269	<1e-26**
	Maltose	0.33±0.09		
	Raffinose	0.29±0.07		
	Starch	0.24±0.05		
	Sucrose	0.3±0.09		
	Xylose	0.41±0.12		
LAB	Fructose	0.34±0.11	12.11	<2e-36 ***
	Maltose	0.29±0.09		
	Raffinose	0.25±0.07		
	Starch	0.23±0.07		
	Sucrose	0.31±0.12		
	Xylose	0.38±0.15		
Yeast	Fructose	0.21±0.15	4.499	<2e-11 ***
	Maltose	0.35±0.15		
	Raffinose	0.15±0.09		
	Starch	0.13±0.10		
	Sucrose	0.33±0.14		
	Xylose	0.19±0.15		
Mold	Fructose	0.34±0.11	4.72	1.39e-11 ***
	Maltose	0.29±0.08		

	Raffinose	0.22±0.10		
	Starch	0.19±0.10		
	Sucrose	0.28±0.14		
	Xylose	0.25±0.12		

\* Significant: Values are presented as means±standard deviation from 6 independent experiments.

Values in a column followed by asterisk are indicates significant differences at  $P < 0.05$ .

#### **4.3.3. Temperature stress test result**

Figure 8 barplot represents the temperature stress test result on 15°C, 25°C, 35°C, 45°C and 55°C. Based on the results of the temperature stress test, it was found that the isolates of both LAB and AMB grew well at temperatures of 25°C and 45°C. However, their growth was much slower at 15°C and 55°C. On the other hand, yeast growth showed better growth at 35°C than 45°C and 55°C. Mold grew well as the temperature gets 35°C and the growth of molds going to slow at 45°C and 55°C (appendix 2 ).

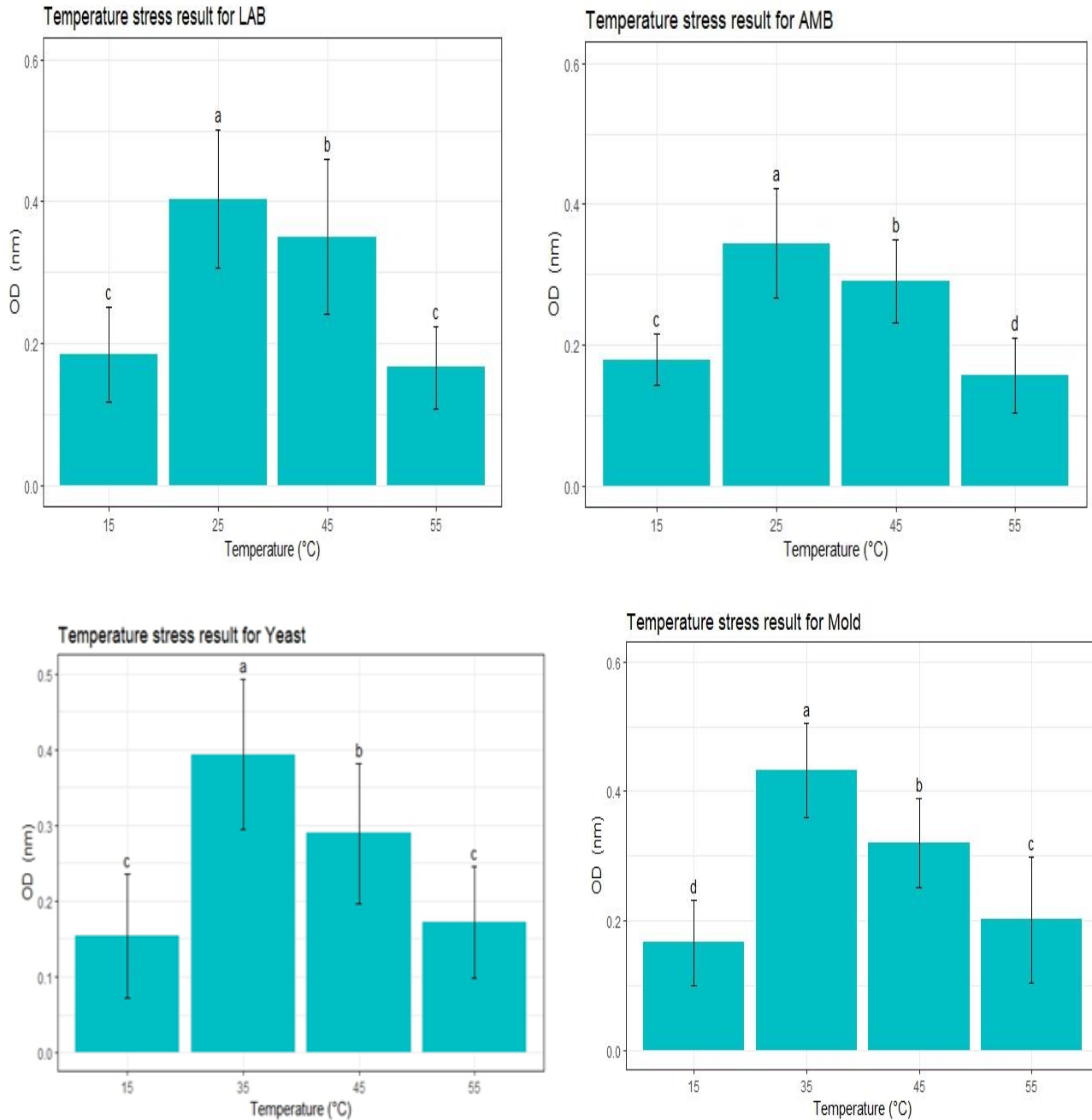


Figure 7. Barplot and tukey test result of LAB, AMB, yeast and mold growth at different temperatures:

Letters show a comparison of isolates growth means per OD medium at the 95% confidence level. The height of the bar represents the mean value, Number of isolates = 3, A significant level ( $P < 0.05$ ). The different letters (a–d) indicate the statistically significant difference at 95% between treatments.

#### 4.3.4. pH stress test

Figure 9 shows that the growth of LAB, AMB, yeast and mold isolates were significantly affected by the pH stress test. Based on the conducted pH test, the highest growth of bacteria (LAB and AMB) were observed at pH 4. At pH 3 & 2 the growth of both bacteria were shown declined. Effect of pH on the growth of yeast and mold were shown in figure 9. The highest growth of both selected microbes were observed when they were cultivated at pH 4 than pH 3 & 2 (appendix 3)

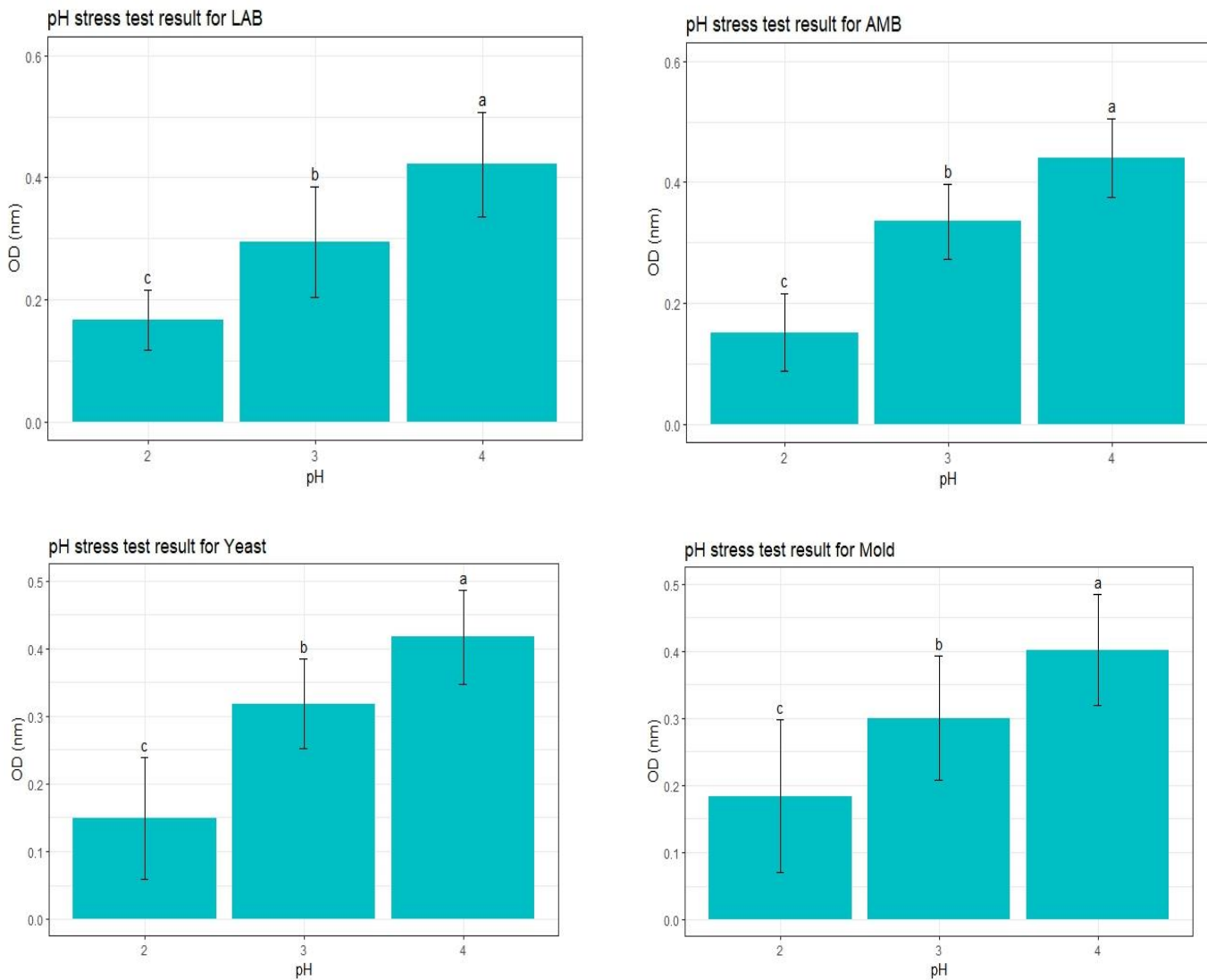


Figure 8. Barplot and tukey test result of LAB, AMB, yeast and mold growth at different pH stress test:

Letters show a comparison of isolates growth means per OD medium at the 95% confidence level.

The height of the bar represents the mean value, Number of isolates = 3, A significant level ( $P <$

0.05). The different letters (a–d) indicate the statistically significant difference at 95% between treatments.

#### **4.3.5. Hydrogen sulfide test**

Result of test for H<sub>2</sub>S production revealed that all isolates of LAB, AMB, yeast, and mold were negative (Figure 10). This means that none of these organisms produced hydrogen sulfide.

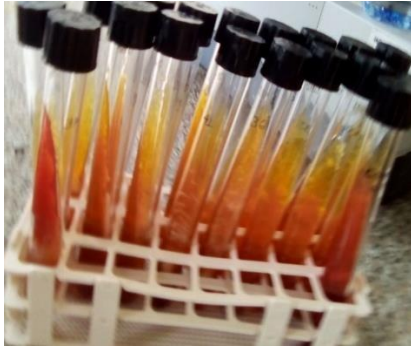


Figure 9. Hydrogen sulfide test result

No blackening of media in any area, control =at right side the first tube

#### **4.4. Performance of selected isolates**

Best performed isolates were obtained by comparing their means based on sugar utilization test, temperature stress test and pH stress test. Furthermore, based on viability testing, the best performing isolates from each group of microbes were chosen and no H<sub>2</sub>S production was detected. Combining all the results, two LAB, one AMB, two yeast and one mold were selected to be used for starter culture formulation.

#### **4.5. Compatibility test result**

The compatibility test experiment was done among LAB, AMB, yeast and mold isolates, none of the isolates showed any inhibitory effects on the other isolates (Figure 11). There were no inhibition zones between the tested isolates.

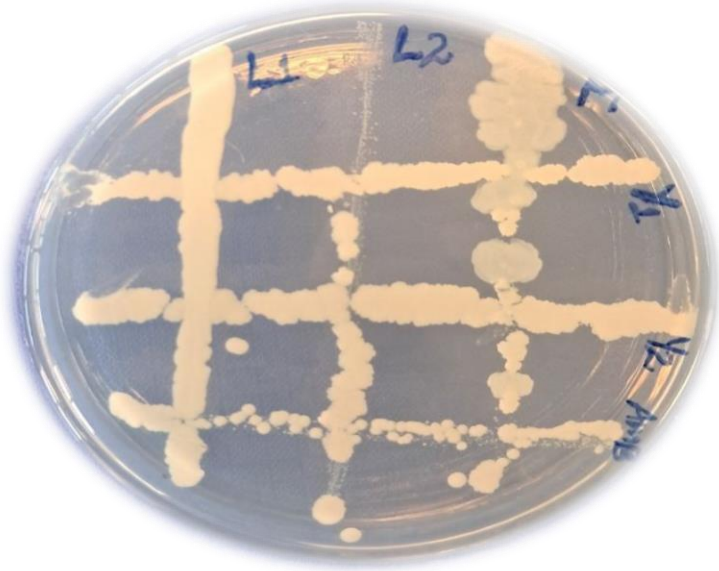


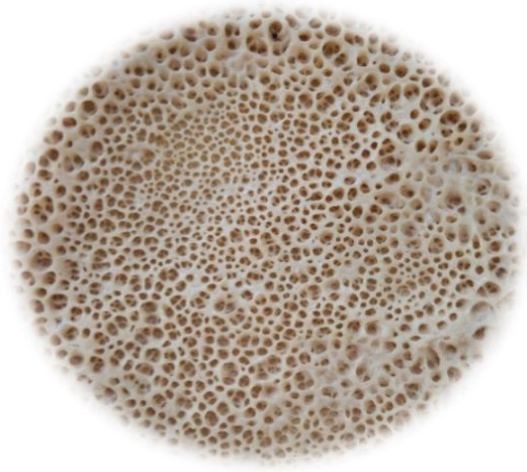
Figure 10 . Compatibility test result

L1 & L2 = LAB, Y1 & Y2 = Yeast, AMB= aerobic mesophilic bacteria, M=Mold

## 4.6. Starter culture formulation

### 4.6.1. Dough fermentation and baking

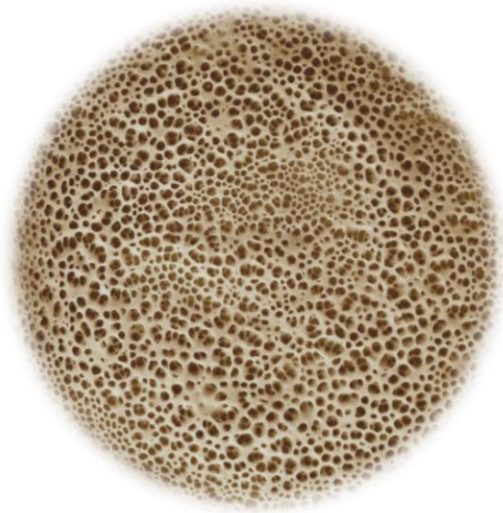
A total of 2 LAB, 1 AMB, 2 yeast and 1 mold isolates performed were by their means values of tolerance with sugar utilization, temperature stress and pH stress test. Out of 59 possible combination (Appendix 2) produced, 29 best performing microbial formulates were selected based on their ability to reduce pH (less than 4.6) (Appendix 3). In addition, the *injera* was separated based on factors such as flavor, texture, uniformity of eye, backside appearance and overall acceptability, using duplicates of 29 fermented dough produced from different microbial formulates. Four of these microbial combinations (treatments) were chosen for final evaluation with 4% inoculum. In terms of pH reduction and *injera* quality (taste, texture, uniformity of eye, backside appearance, and overall acceptability), the *teff dough* made from 4% inoculum of the formulation was better than that made from 1%, 2%, and 3% inoculums of the formulations. It was found that 28 hrs were required for the first and second fermentations of *injera* with 4% inoculum. Figure 12 depicted that formulated starter formulations (T1- T4) produced better quality of *injera* with respect to taste, texture, uniformity of eye, backside appearance, and overall acceptability after 28 hrs of fermentation among rest combination.



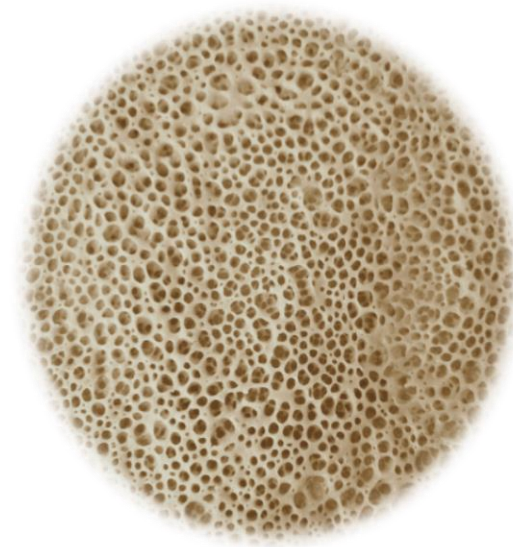
T1. L2 + Y1 + Y2 + M + AMB



T2. L1 + L2 + Y1 + Y2 + AMB



T3. L1 + Y1 + Y2 + M + AMB



T4. L1+L2+Y1+Y2+AMB+M

Figure 11. The four *injera* products prepared from four types of formulates.

Starter formulations (T1, T2, T3 and T4) produced better quality of *injera*

#### **4.7. pH measurement during fermentation**

Variations in pH during the formulated starter culture fermentation of *teff* batter were measured at 12, 26 and 28 hrs. Changes in pH were distinct on the basis of microbial load and composition in the formulation. When the *teff* batter reached the end of its fermentation (at 28 hrs of baking), its pH decreased and ranged from 3.7 to 4.6 (Appendix 4). Out of 59 formulates 29 best performing formulates having pH less than 4.6 were selected and *injera* was made from each dough of formulation after 28 hrs of fermentation (Appendix 4).

#### **4.8. Sensory evaluation result**

In the current study, a panel of 10 judges was used to describe the degree of consumer acceptance and satisfaction to the *injera* prepared using different combinations of microbes. Combinations of microbes T4 (L1+L2+Y1+Y2+AMB+M) was the most well-liked of the four *injera* samples in terms of taste, texture, quality of eyes, odour, backside appearance and overall acceptability based on panelists response and T3 (L1+Y1+Y2+AMB+M1) which comes after *injera* sample T4 (Table 2).

Table 2. Distribution of responses on a hedonic scale of 1–5 (dislike to like), with resulting statistical indices for the four *injera* types testing for taste, texture, odour, quality of eyes, backside appearance, and overall acceptance.

Assigned value		Frequency of responses				
		T1	T2	T3	T4	Control
<i>Injera</i> types		T1	T2	T3	T4	Control
pH		3.8	3.78	3.85	3.70	4.1
<b>Taste</b>						
Dislike very much	1	0	0	0	0	0
Dislike moderately	2	2	0	1	0	0
Neither like nor dislike	3	2	3	3	0	1
Like moderately	4	5	6	4	2	6
Like very much	5	1	1	2	8	3
Total		10	10	10	10	10
Mean		3.5 <sup>b</sup>	3.8 <sup>b</sup>	4 <sup>ab</sup>	4.8 <sup>a</sup>	4.2 <sup>ab</sup>
<b>Texture</b>						
Dislike very much	1	0	0	0	0	0
Dislike moderately	2	1	0	0	0	0
Neither like nor dislike	3	4	4	1	0	1
Like moderately	4	3	4	6	6	6
Like very much	5	2	2	3	4	3
Total		10	10	10	10	10
Mean		3.6 <sup>b</sup>	3.8 <sup>ab</sup>	4.2 <sup>ab</sup>	4.6 <sup>a</sup>	4.2 <sup>ab</sup>
<b>Quality of eye</b>						
Dislike very much	1	0	0	0	0	0
Dislike moderately	2	0	0	0	0	0
Neither like nor dislike	3	3	6	1	1	2
Like moderately	4	5	3	6	5	6
Like very much	5	2	1	3	4	2

Total		10	10	10	10	10
		3.5 <sup>a</sup>	3.9 <sup>a</sup>	4.2 <sup>a</sup>	4.3 <sup>a</sup>	4 <sup>b</sup>
<b>Backside appearance</b>						
Dislike very much	1	0	0	0	0	.0
Dislike moderately	2	2	0	0	0	0
Neither like nor dislike	3	6	7	2	0	1
Like moderately	4	2	3	5	5	5
Like very much	5	0	0	3	5	4
Total		10	10	10	10	10
Mean		3 <sup>b</sup>	3.3 <sup>b</sup>	4.1 <sup>a</sup>	4.5 <sup>a</sup>	4.3 <sup>a</sup>
<b>Odour</b>						
Dislike very much	1	0	0	0	0	0
Dislike moderately	2	0	0	0	0	0
Neither like nor dislike	3	5	3	2	0	2
Like moderately	4	5	6	7	5	7
Like very much	5	0	1	1	5	1
Total		10	10	10	10	10
Mean		3.5 <sup>b</sup>	3.8 <sup>b</sup>	3.9 <sup>ab</sup>	4.5 <sup>a</sup>	3.9 <sup>ab</sup>
<b>Overall acceptability</b>						
Dislike very much	1	0	0	0	0	0
Dislike moderately	2	1	0	0	0	0
Neither like nor dislike	3	4	4	0	0	0
Like moderately	4	5	6	8	5	7
Like very much	5	0	0	2	5	3
Total		10	10	10	10	10
Mean		3.4 <sup>c</sup>	3.6 <sup>bc</sup>	4.2 <sup>ab</sup>	4.5 <sup>a</sup>	4.3 <sup>ab</sup>

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The letters (a,b and c) represent the different statistical groups

**NB:** T1= L2 + Y1 + Y2 + M + AMB    T2= L1 + L2 +Y1 + Y2 + AMB

T3= L1 + Y1 + Y2 + M + AMB    T4= L1+L2+Y1+Y2+AMB+M

Radar plot for sensory evaluation shows that, in comparison to T1, T2, and T3, the T4 *injera* mean value was approaching five (5), highest mean. In terms of taste, texture, quality of eyes, odour, backside appearance and overall acceptability (Figure 11).

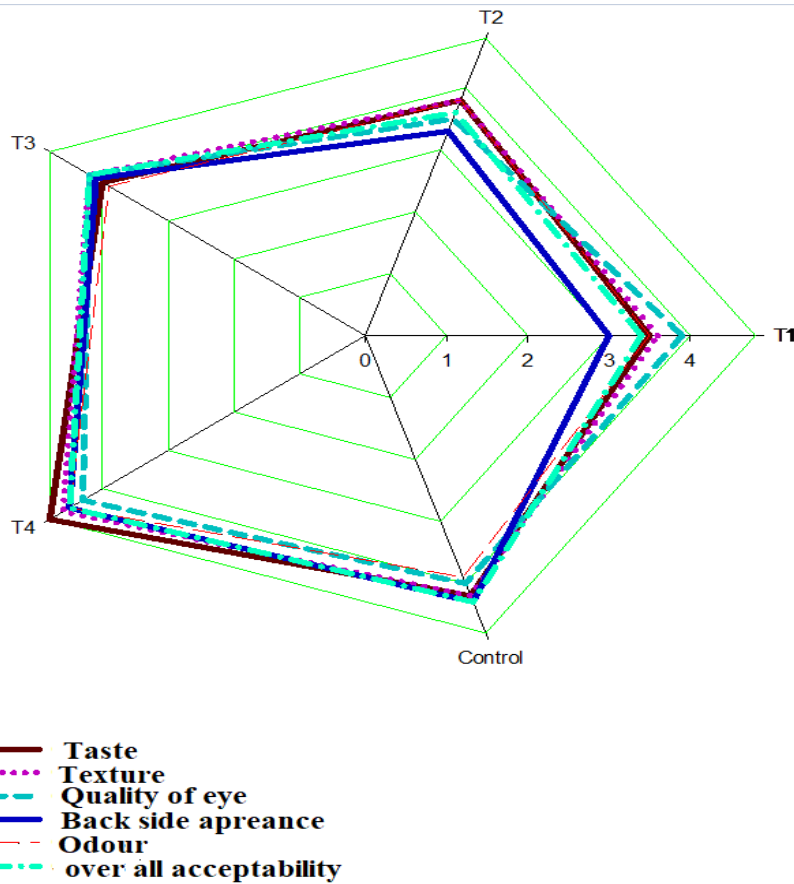


Figure 12. Radar plot of the four sensory attributes of *injera*

#### 4.9. Microbial dynamics

The numbers of both lactic acid bacteria and aerobic mesophilic bacteria were increased rapidly at early stage (0–12 hr) of fermentation. As the fermentation progressed, the populations of AMB were began to decrease, while the populations of yeasts, mold, and LAB continued to rise (Figure 14).

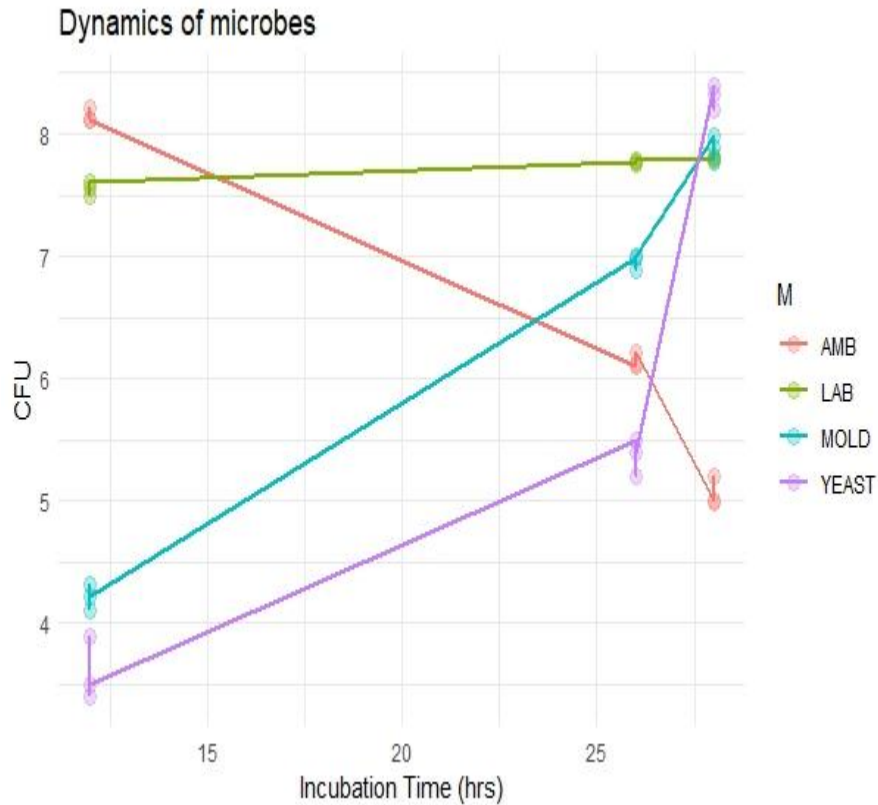


Figure 13. Microbial dynamics during *teff injera* fermentation

**Note:** AMB=Aerobic Mesophilic bacteria, LAB=Lactic Acid Bacteria

#### 4.10. Molecular identification of the selected microbes

##### 4.10.1. PCR amplifications of LAB and AMB

The bacterial isolate and fungi genomic DNA were extracted and amplified by polymerase chain reaction (PCR) and visualized using agarose gel electrophoresis. The bacterial isolates had PCR amplified fragments with primer pairs (RD1/FD1) of about 1500 bp size (Figure 15).

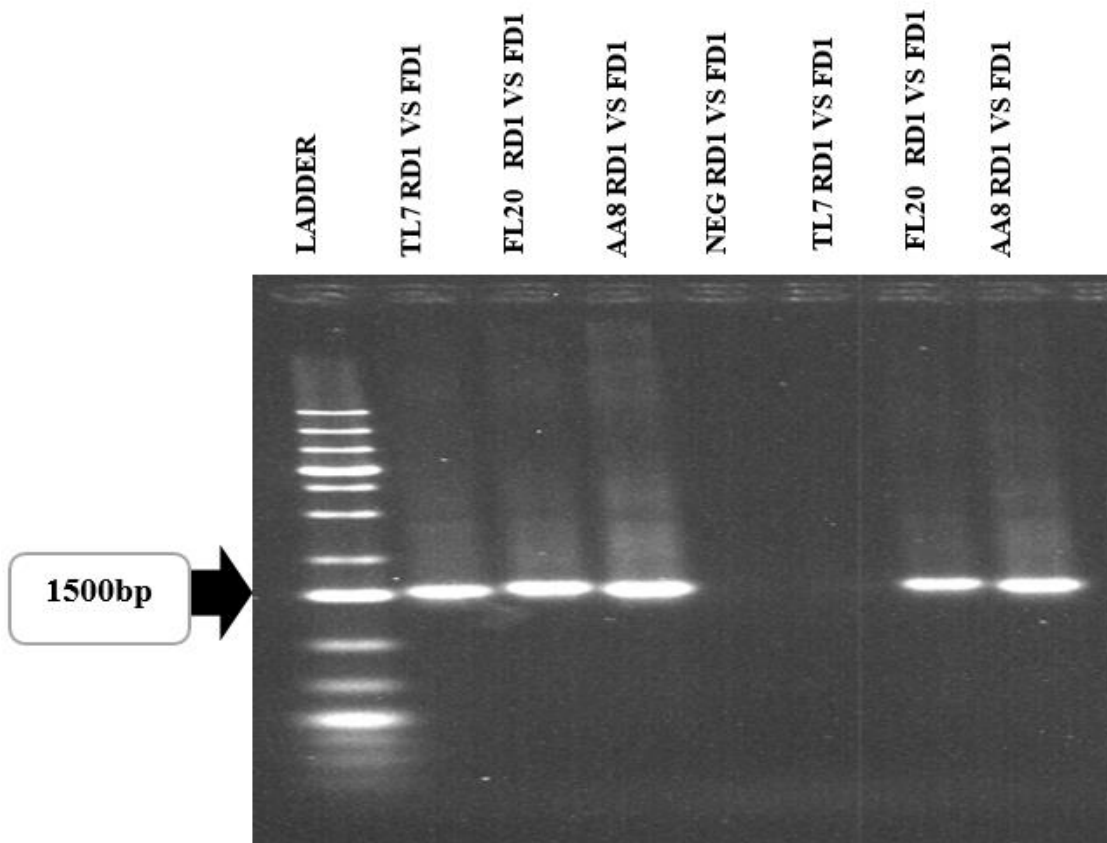


Figure 14. PCR amplification 16s rRNA using rD1 and fD1 bacterial universal primers, 1kb DNA ladder, amplicon size; 1500bp

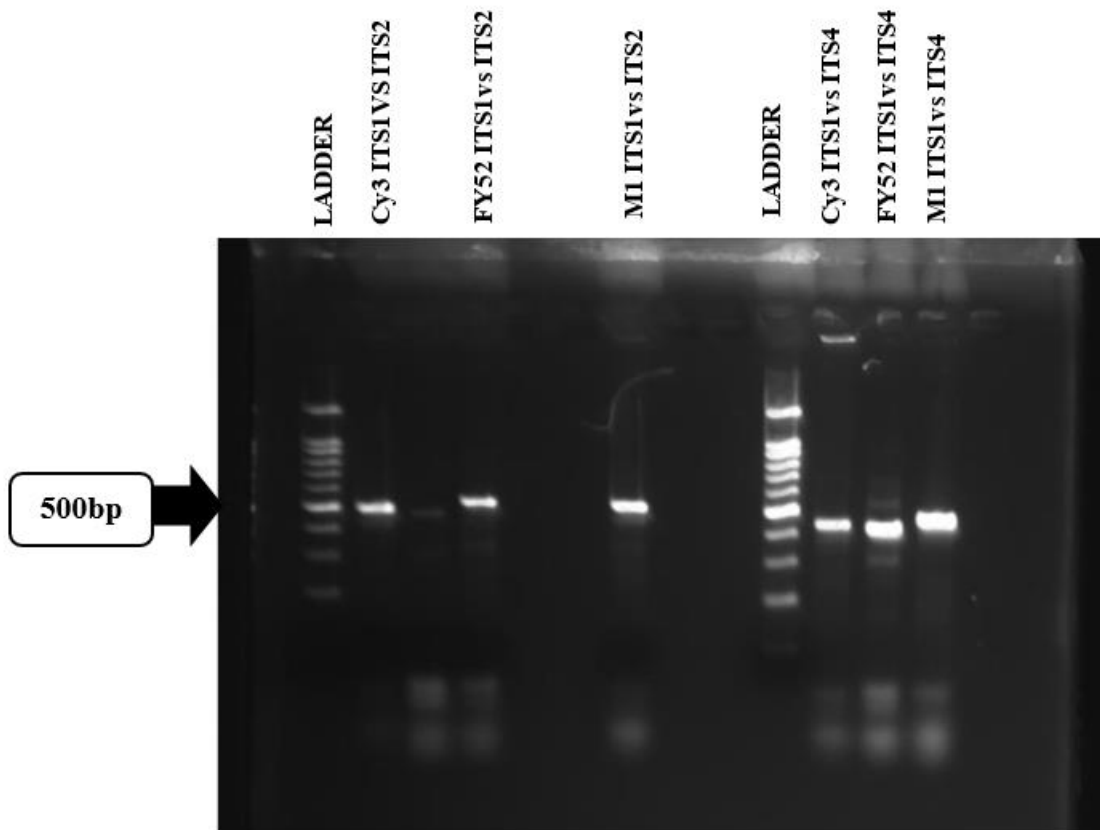


Figure 15. The PCR amplification product of yeast and mold isolates with ITS primers between 400-500bp , fragment length of ladder is 100bp

#### 4.10.2. The Sequence analyses of LAB and AMB

Table 3 depicted the identification of number of bacterial isolates those were included in the formulated starter culture on the basis their 16S rDNA sequences with NCBI in the GenBank and on the basis of their similarity to strains from the GenBank with their different bacterial accessions. Accordingly, the three bacterial isolates were found to belong to three genera in which LAB were L2 was identified as *Lacticaseibacillus paracasei* strain PBM7 and *Lentilactobacillus parabuchneri* strain ML19. Similarly, isolates (L1) was identified as *Bacillus subtilis* strain SB-1, specifically strains 154.1. AMB identified was *Bacteruim* strain ANA YJ H29 (Figure 17).

Table 3. Phylogenetic relationship of LAB and AMB isolates

Isolates included in the starter formulation					
No	Sequence ID	E-value	Identity	Species	Accession
1	L1	0.0	97%	<i>Bacillus subtilis</i> strain SB-1	OR342736.1
2	AMB	0.0	99.8%	<i>Bacterium</i> strain ANA YJ E80	MW037500.1
3	L2	0.0	97%	<i>Lentilactobacillus parabuchneri</i> strain	MW595791.1

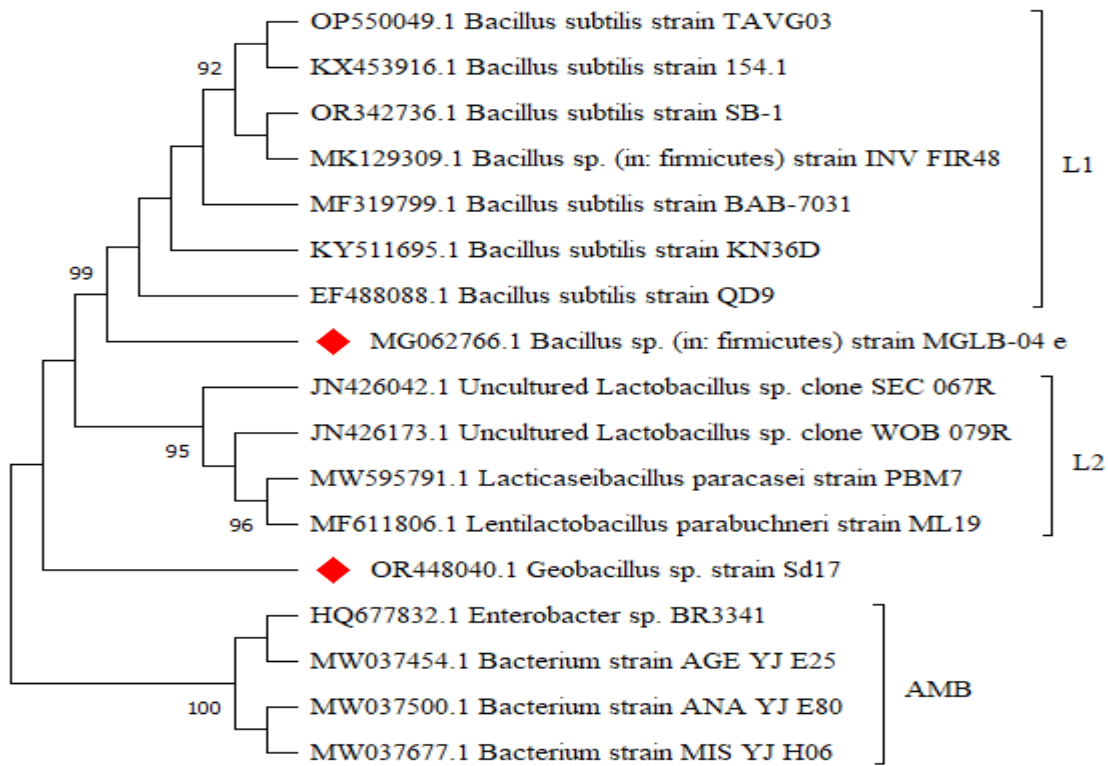


Figure 16. Phylogenetic relationship of LAB and AMB

#### 4.10.3. The Sequence analyses of yeast

Table 4 depicted the identification of number of yeast isolates those were included in the formulated starter culture on the basis their ITS DNA sequences with NCBI in the GenBank and on the basis of their similarity to strains from the GenBank with their different yeast accessions. On basis of the phylogenetic tree both yeasts Y1 and Y2 belongs strains of *P.fermentans* ATCC and *P.fermentans* internal transcribed spacer 1 respectively.

Table 4. Phylogenetic relationship of yeast isolates

Isolates included in the starter formulation					
No	Sequence ID	E-value	Identity	Species	Accession
1	Y1	0.0	97.5%	<i>P.fermentans ATCC</i>	AF336843
2	Y2	0.0	98.3%	<i>P.fermentans internal transcribed spacer 1</i>	DQ646687

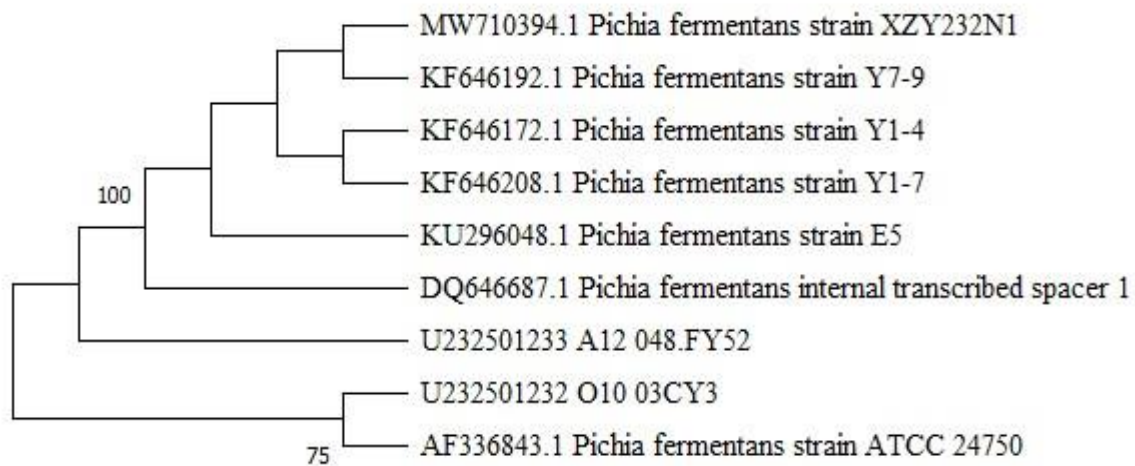


Figure 17. Phylogenetic relationship of yeasts

#### 4.10.4. The Sequence analyses of mold

Table 5 depicted the identification of number of mold isolate this was included in the formulated starter culture on the basis their ITS DNA sequences with NCBI in the GenBank and on the basis of their similarity to strains from the GenBank with their different mold accessions. On basis of the phylogenetic tree M1 was belongs to *Aspergillus niger* strain.

Table 5. Phylogenetic relationship of mold isolate

Isolates included in the starter formulation					
No	Sequence ID	E-value	Identity	Species	Accession
1	M	0.0	99%	<i>Aspergillus niger</i> isolate MEBP004	MT5978234.1

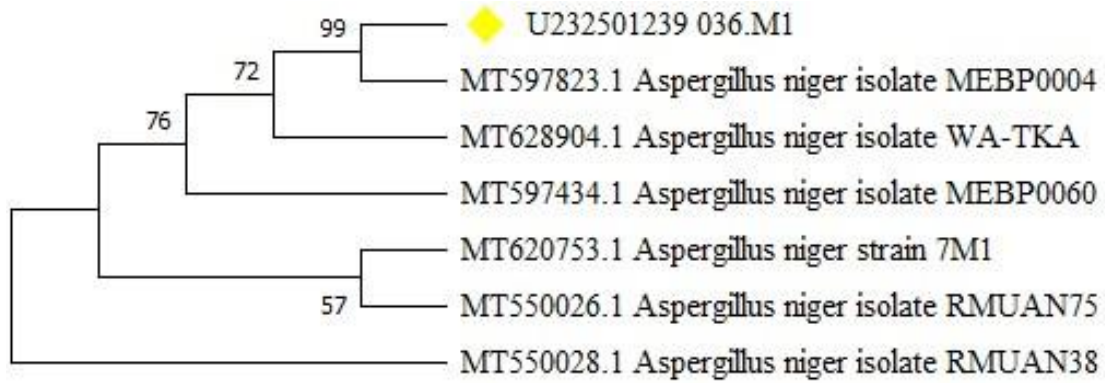


Figure 18. Phylogenetic relationship of mold

## 5. Discussion

The microbes such as lactic acid bacteria, aerobic mesophilic bacteria, yeast, and mold were successfully recovered and identified based on colonial and morphological characteristics. Zewdie *et al.*, (1997) reported that the predominant organisms identified were Lactobacillus, Bacillus and Yeasts. In the preliminary screening, lactic acid bacteria and aerobic mesophilic bacteria were selected based on the change in color from red to yellow. Tests were conducted on purified isolates to see if they used sugar, producing acidic products, or turning the sugar solution yellow (Lee, 2021). Lactic acid bacteria and aerobic mesophilic bacteria were selected based on the change in color from red to yellow. Yeast isolates were chosen based on their ability to produce active gas from glucose. It was thought that yeast isolates with good potential for gas production were considered good fermenters and crucial to the formation of numerous eyes on *injera* that was ready to consume. Additionally, ethanol, carbon dioxide, and other byproducts are yeast fermentation secondary products, which improve the final product's flavor, taste, and quality (Lilly *et al.*, 2000).

Based on the information provided, it is clear that temperature and pH have a significant impact on the growth of microorganisms such as bacteria, yeast, and mold (Hadinia, *et al.*, 2022). Both aerobic mesophilic bacteria and lactic acid bacteria (LAB) were found to be able to grow at stressful temperatures, and their growth was shown to be better at 25°C than it was at 45°C (Figure 8). These results were consistent with previous reports that LAB growth is lower at higher temperatures, resulting in less acid production (Hadinia *et al.*, 2022). Among other temperatures, strains of mold and yeast could grow well at 35°C (Figure 8). Karki *et al.* (2017) reported findings that were comparable.

The pH of the medium also plays a significant role in the growth of microorganisms. LAB showed optimum growth at pH 4, with reduced growth observed at pH 3 and 2 (Figure 9). These results were found similar to that reported in the study done by (Vera-Peña and Rodriguez, 2020). Yeast strains from this study were also exhibited different growth patterns at different pH conditions, with optimal growth observed at pH 4 (Yalcin and Ozbas, 2008) and some tolerance to pH 3 and 2 (Figure 9). The ability of yeast to grow well below pH 4.0 helps reduce loss brought on by bacterial contaminants in non-aseptic equipment (Hettenhaus, 1998). The optimal mold growth

was observed at pH 4 rather than pH 3 and 2. Mold, showed higher growth rates range from slightly acidic to neutral pH conditions (Miranti *et al.*, 2018).

The findings of this study showed that all of the selected isolates have the capacity to utilize examined sugar types. It can be seen from the results obtained LAB and AMB play vital role in degrading of raffinose and starch. It seems that different types of lactic acid bacteria and AMB can metabolize different carbohydrates, which has implications for their potential use in the food industry (Velikova *et al.*, 2016). Additionally, yeasts and molds are able to utilize mono- and disaccharides sugars rather than polysaccharides. These findings align with the result reported by (Thais *et al.*, 2006).

Starter cultures were selected based on their safety and functional qualities. However, they must be able to tolerate stress and adjust to harsh environments before they can be utilized in industrial conditions (Saarela *et al.*, 2000). In the case of *injera* making, isolates that were able to tolerate stress and adapt to extreme conditions were selected as potential starter cultures. After conducting stress tests, the best-tolerated isolates from each group of microbes (lactic acid bacteria, aerobic mesophilic bacteria, yeast, and mold) were selected. Their viability was then checked at different temperatures and pH levels. Results of this study showed that all selected isolates have the potential to grow at all tested temperature values, better at 35°C and 45°C, than at 15°C and 55°C. Similarly, the test of growing at lower pH values exhibited that all tested isolates have the potential to grow and survive in acidic environment. Under environmental stress conditions, LAB change the synthesis of enzymes and metabolites to adapt the metabolism of carbon sources to the new environment, change growth rate, regulate energy and metabolic fluxes (Koponen *et al.*, 2012). Additionally, optimizing fermentation conditions to encourage the growth and activity of aerobic mesophilic bacteria, which will result in fermented foods that are more reliable and of higher quality (Gaglio *et al.*, 2015).

Starter cultures are single or combined formulations of selected microbe that have a unique capacity to convert a substrate into a food product with particular properties when added in specific amounts (Hammes and Hertel, 1998; Laranjo *et al.*, 2017). In this study, two LABs, one AMB, two yeasts, and one mold isolate were selected on the basis of their best performance according to the selection criteria . The greatest scores from the temperature stress test, the acid stress test, and

the sugar utilization test were combined to choose each of the six strains that were ultimately chosen. Furthermore, the production of hydrogen sulfide and their viability were also utilized. In this study, four treatment groups were designed, and 59 combinations were made using the best six selected microbial isolates (Appendix 3). These isolates represented each respective group (LAB, yeast, mold, and aerobic mesophilic bacteria) were used in the formulation of the starter culture.

The results of the sensory evaluation showed that all four types of *injera* prepared with different combinations of microbes were well-liked by the panelists. The taste, texture, quality of the eye, backside appearance, and overall acceptability of the *injera* were evaluated, and all treatments indicating that they were considered good products. The most preferred *injera* was produced using a combination of L1, L2, Y1, Y2, AAB, and M1 microbes (included in T4). This combination resulted in the highest ratings for all sensory attributes. The produced *injera* requires 28 hours for both first and second fermentations, with the best combination (T4). The rapid growth of LAB reduces pH and favors yeast growth in the batter fermentation stage that follows (Gobbetti, 1998). The current study findings are consistent with those reported by (Holzapfel, 2002) who indicated that the rapid acidity and flavor development happen quickly as a result of starter cultures growing quickly. It is worth noting that fermentation not only improves the sensory characteristics of *injera*, such as flavor, aroma & color, but also enhances its nutritional content. Overall, the study suggests that the rapid growth of starter cultures during fermentation contributes to product safety, as well as flavor development. The sensory evaluation confirmed that the *injera* produced with the T<sub>4</sub> combination of microbes was the most preferred by the panelists.

Molecular identification of bacteria from formulated starter culture isolates by using sequence data identified were *Bacillus subtilis* SB-1 strain, *Lactobacillus paracasei* PBM7 strain, *Bacterium* strain AGE YJ E29. These findings align with the result reported by (Tadesse *et al.*, 2019) *Enterococcus avium*, *Enterococcus durans*, *Lactobacillus paracasei*, *Enterococcus hirae*, *Lactobacillus brevis*, *Enterococcus faecium*, and *Bacillus subtilis* were identified fermenting *teff* dough. Enzymes that break down carbohydrates, such as amylase, galactanase, galactosidase, glucosidase, and fructofuranosidase, are produced by *Bacillus* species (Aderibigbe and Odunfa, 1990; Sarkar *et al.*, 1997; Omafuvbe *et al.*, 2000; Kiers *et al.*, 2000). *L. buchneri* MF58 displaying the highest phytic acid degrading potential (Neela and Fanta, 2020). The yeast isolates were

identified as belonging to strain of *Pichia fermentans* strain. Yeast is responsible for *injera* fermentation also they identified *Pichia fermentans*, *Pichia occidentalis*, *Candida humilis*, *Saccharomyces cerevisiae*, and *Kazachstania bulderi* species in fermented *injera* batter (Tadesse *et al.*, 2019). Mold isolate belonging to *Aspergillus niger* strain. *A. niger* synthesizes amino acids and different protein hydrolysis to create flavorings and active ingredients (Gao *et al.*, 2018; Zhao *et al.*, 2017 and Park *et al.*, 2017).

## 6. Conclusion and recommendation

### 6.1. Conclusion

Traditional fermented foods are ones that are native to a certain region and were created there by the local people with traditional methods and ingredients that were readily available raw material. *Injera* is one of these traditional fermented food products, which is mainly produced from the flour of *teff* seeds. It is a basic food that is consumed on a daily basis in almost every Ethiopian home. The raw ingredients used for preparation of *injera* are *teff* flour, water and *ersho*. The present study The fermentative microbes (lactic acid bacteria, aerobic mesophilic bacteria, yeast, and mold) from *teff ersho* with the highest fermentative potential were isolated and characterized The *injera* produced with these microbes had positive sensory attributes similar to traditional *injera* but also had the advantages of shorter fermentation time and reduced pH value. This can help reduce wastage of time during the fermentation process.

The study also provided information on selection of starter cultures for the production of consistent quality *injera* with different sensory qualities. *Teff injera* produced by combining all four microbial groups were acceptable by panelists. The use of these isolates as starter was observed beyond reducing the fermentation time and enhancing the overall qualities of *injera* including its sensory attributes, it may be used to standardize, ensuring the persistence, extend the shelf life and large scale production of the final ready-to-consume product of the product. According to the study, *teff ersho* was caused by a variety of bacteria, yeast and mold. The identification of these isolates could possibly contribute to the information needed for understanding and verification of potential microbes involved in the course of *teff ersho* fermentation.

## 6.2. Recommendation

For Ethiopians, *injera* is a traditional and staple fermented food item. It was still prepared *following* age-old traditional techniques. This study is part of a grand project that aimed at transforming the biotechnology of *injera* production to a large scale production by standardizing raw materials, standardizing processing techniques, ensuring the qualities of *injera*, extending the shelf-life, and producing starter culture. In line with this, as *erisho* is 'undefined traditional 'starter culture' used for back slopping and this study was concerned with isolating, screening, and selecting the best fermentative microbes to formulate a potential starter culture. The following are recommendation for further study.

- Further analysis of the profile and performance of the selected starter culture.
- Analysis of the shelf life of the selected starter culture.
- Packaging of formulated starter culture for commercial purposes and promoting its production is recommended.

## 7. Limitation of the study

Limitations during the research process arise from:

- The collection of the sample were difficult due unexsistence of *erisho* at arrival time
- Obtaining of some chemicals has been challenging

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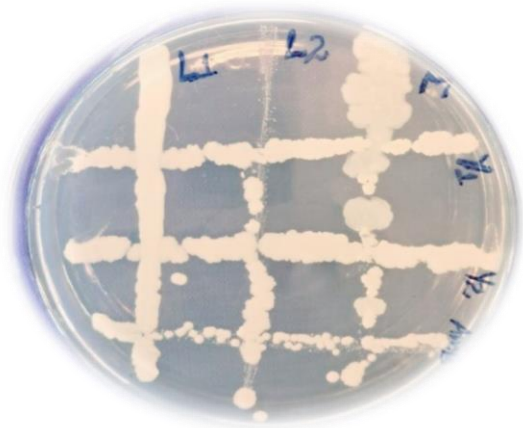
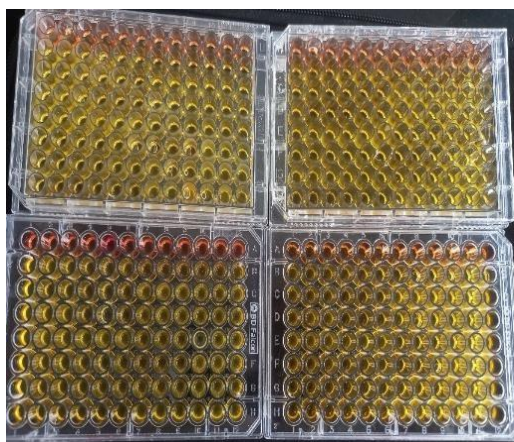
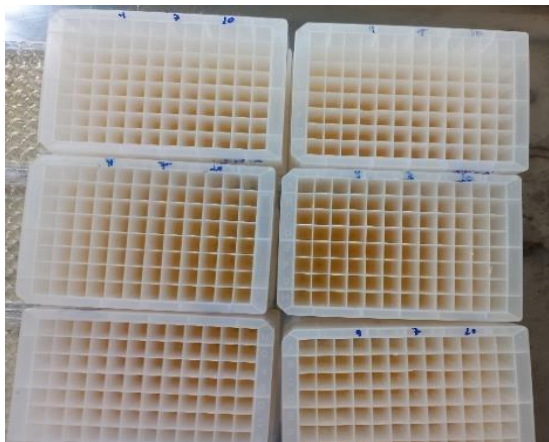
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# Appendix

## Appendix 1: Screening tests



## Appendix 2. Temperature stress test result

Table 6. Temperature stress test result for LAB, AMB, yeast and mold

Types of microbes	Temperature	Mean±sd	f- value	p - value
LAB	15°C	0.184±0.066	2.852	1.03e-07 ***
	25°C	0.403±0.096		
	45°C	0.350±0.108		
	55°C	0.166± 0.057		
AMB	15°C	0.179±0.036	1.68	0.00373 **
	25°C	0.344±0.077		
	45°C	0.290±0.059		
	55°C	0.157±0.052		
Yeast	15°C	0.153±0.081	4.591	<2e-16 ***
	35°C	0.393±0.099		
	45°C	0.289±0.092		
	55°C	0.171±0.073		
Mold	15°C	0.166±0.065	1.69	0.0275 *
	35°C	0.431±0.072		
	45°C	0.32±0.069		
	55°C	0.201±0.096		

### Appendix 3. pH stress test result

Table 7. pH stress test result for LAB, AMB, yeast and mold

Types of microbes	pH	Mean±sd	f- value	p - value
LAB	2	0.188±0.048	1.862	0.00209 **
	3	0.326± 0.326		
	4	0.473±0.083		
AMB	2	0.152±0.064	1.321	0.0827
	3	0.335±0.061		
	4	0.44±0.074		
Yeast	2	0.149±0.091	1.783	0.000866 ***
	3	0.317±0.066		
	4	0.416±0.069		
Mold	2	0.185±0.115	5.287	2.02e-11***
	3	0.299±0.092		
	4	0.37±0.085		

## Appendix 4: Stages of injera

*Teff* flour



Mix with water and starter culture then knead



Vigorous gas evolution and maximum dough expansion



Yellowish liquid on dough surface then discard it  
*Absit* mixed back to the fermented dough and baked



Bake on hot greased clay griddle (metad)



## Appendix 5

Table 8: Combination of selected isolates

<b>Isolate 1</b>	<b>No</b>	<b>Treatment one (1)</b>
LAB1	1.	LAB1
	2.	L1 + L2
	3.	L1 + Y1
	4.	L1 + Y2
	5.	L1 + M
	6.	L1 + AMB
	7.	L1 + L2 + Y1
	8.	L1 + L2 + Y2
	9.	L1 + L2 + M
	10.	L1 + L2 + AMB
	11.	L1 + Y1 + Y2
	12.	L1 + Y1 + M
	13.	L1 + Y1 + AMB
	14.	L1 + Y2 + M
	15.	L1 + Y2 + AMB
	16.	L1 + M + AMB
	17.	L1 + L2 + Y1 + Y2
	18.	L1 + L2 + Y1 + M
	19.	L1 + L2 + Y1 + AMB
	20.	L1 + L2 + Y2 + M
	21.	L1 + L2 + Y2 + AMB
	22.	L1 + L2 + M + AMB

	23.	$L1 + Y1 + Y2 + M$
	24.	$L1 + Y1 + Y2 + AMB$
	25.	$L1 + L2 + Y1 + Y2 + M$
	26.	$L1 + L2 + Y1 + Y2 + AMB$
	27.	$L1 + Y1 + Y2 + M + AMB$
	28.	$L1 + L2 + Y1 + Y2 + M + AMB$
LAB2	1.	$L2$
	2.	$L2 + Y1$
	3.	$L2 + Y2$
	4.	$L2 + M$
	5.	$L2 + AMB$
	6.	$L2 + Y1 + Y2$
	7.	$L2 + Y1 + M$
	8.	$L2 + Y1 + AMB$
	9.	$L2 + Y2 + M$
	10.	$L2 + Y2 + AMB$
	11.	$L2 + M + AMB$
	12.	$L2 + Y1 + Y2 + M$
	13.	$L2 + Y1 + Y2 + AMB$
	14.	$L2 + Y1 + M + AMB$
	15.	$L2 + Y2 + M + AMB$
	16.	$L2 + Y1 + Y2 + M + AMB$

<b>Isolate 2</b>	<b>No</b>	<b>Treatment two (2)</b>
Yeast 1	1.	Y1
	2.	Y1 + Y2
	3.	Y1 + M
	4.	Y1 + AMB
	5.	Y1 + Y2 + M
	6.	Y1 + Y2 + AMB
	7.	Y1 + M + AMB
	8.	Y1 + Y2 + M + AMB
Yeast2	1.	Y2
	2.	Y2 + M
	3.	Y2 + AMB
	4.	Y2 + M + AMB

<b>Isolate 3</b>	<b>No</b>	<b>Treatment three (3)</b>
Mold	1.	M
	2.	M + AMB

<b>Isolate 4</b>	<b>No</b>	<b>Treatment four (4)</b>
AMB	1.	AMB

## Appendix 6:

Table 9: pH measurement

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1	L1 + L2 + Y1	At baking (28hrs)	4.25
2	L1 + L2 + Y2	At baking (28hrs)	4.20
3	L1 + L2 + M	At baking (28hrs)	4.31
4	L1 + L2 + AMB	At baking (28hrs)	3.93
5	L1 + Y1 + Y2	At baking (28hrs)	4.30
6	L1 + Y1 + M	At baking (28hrs)	4.33
7	L1 + Y2 + AMB	At baking (28hrs)	4.10
8	L1 + M + AMB	At baking (28hrs)	4.25
9	L1 + L2 + Y1 + Y2	At baking (28hrs)	4.20
10	L1 + L2 + Y1 + M	At baking (28hrs)	4.22
11	L1 + L2 + Y2 + AMB	At baking (28hrs)	3.98
12	L1 + L2 + Y2 + M	At baking (28hrs)	4.23
13	L1 + L2 + Y2 + AMB	At baking (28hrs)	3.94
14	L1 + L2 + M + AMB	At baking (28hrs)	3.99
15	L1 + Y1 + Y2 + M	At baking (28hrs)	4.21
16	L1 + L2 + Y1 + Y2 + M + AMB	At baking (28hrs)	3.70
17	L1 + Y1 + Y2 + AMB	At baking (28hrs)	4.00
18	L1 + L2 + Y1 + Y2 + M	At baking (28hrs)	3.94
19	L1 + L2 + Y1 + Y2 + AMB	At baking (28hrs)	3.78
20	L1 + Y1 + Y2 + M + AMB	At baking (28hrs)	3.85

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21	L2 + Y1 + M	At baking (28hrs)	4.40
22	L2 + Y1 + AMB	At baking (28hrs)	4.21
23	L2 + M + AMB	At baking (28hrs)	4.31
24	L2 + Y1 + Y2 + M	At baking (28hrs)	4.30
25	L2 + Y1 + Y2 + AMB	At baking (28hrs)	4.22
26	L2 + Y2 + M + AMB	At baking (28hrs)	4.11
27	L2 + Y1 + Y2 + M + AMB	At baking (28hrs)	3.80
28	Y1 + Y2 + M	At baking (28hrs)	4.55
29	Y1 + Y2 + M + AMB	At baking (28hrs)	4.58
30	Control.....	At baking (28hrs)	4.1

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**Appendix 7: Sensory evaluation form**

**Directions:** - Check one rating for each of the following: taste, texture, eye uniformity, odour, backside appearance and overall acceptability was evaluated on a scale from 1 to 5, ‘1’ being “dislike extremely, ‘5’” being “like extremely” to with resulting statistical indices on the *injera* fermented with the formulated starter culture.

Panelist name:-		Product code:-					
		Sensory attribute					
		Texture	Odour	Taste	Eye uniformity	Backside appearance	Overall acceptability
5	Like very much						
4	Like moderately						
3	Neither like nor dislike						
2	Dislike moderately						
1	Dislike very much						

Other note :-----  
 -----  
 -----  
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