ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
DEPARTMENT OF MICROBIAL, CELLULAR AND MOLECULAR BIOLOGY

Microbial Diversity and Probiotic Potential of Lactic Acid Bacteria Isolated from Traditionally Fermented Food Products and their Applicability for Prevention of Selected Foodborne Pathogens

A Thesis Presented to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (PhD) in Biology (Applied Microbiology)

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Addis Ababa, Ethiopia
November, 2019

ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MICROBIAL, CELLULAR AND MOLECULAR BIOLOGY

This is to certify that the Thesis prepared by Guesh Mulaw, entitled: **''Microbial Diversity and Probiotic Potential of Lactic Acid Bacteria Isolated from Traditionally Fermented Food Products and their Applicability for Prevention of Selected Foodborne Pathogens"** and submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy (Applied Microbiology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Probiotics are live microorganisms which when administered in adequate amounts promote the health of the consumer. The major purpose of this study was to determine bacterial diversity of traditionally fermented food products (*teff* dough, *Ergo and Kocho*) and to assess their probiotic potential of lactic acid bacteria in prevention of foodborne pathogens. Culture-independent metagenomic methods, particularly 16S rRNA amplicon sequencing were applied to study the bacterial diversity of some fermented foods. The sequencing of the amplicons resulted in an average of 76,594.33 ± 2,876.25 high-quality reads per sample. The bacterial communities of the three traditional fermented foods were dominated by the phylum Firmicutes, followed by Proteobacteria and Actinobacteria. At the genus level, the prevalent microorganisms were *Lactobacillus*, *Zymomonas*, *Streptococcus*, *Leuconostoc*, *Bacillus* and *Weissella*. The genus *Lactobacillus* was the most abundant genus in the three studied food products, whereas *Zymomonas* was the most dominant genus in fermented *ergo*. In order to assess the probiotic potential of strains isolated from these fermented products, a total of 450 LAB were isolated from the fermented products, and their ability to survive the gastrointestinal conditions was tested. Among them, 34 (7.56%) isolates survived at pH 2 for 3 and 6 h, showing a survival rate of 45.35- 96.33% and 33.33- 91.75%, respectively. All the 34 acid-tolerant isolates survived in the presence of 0.3% bile salts for 24 h with 82.58 to 99.44% rate of survival. The acid and bile salt-tolerant LAB isolates shown inhibiting some bacterial foodborne pathogen into varying degrees. Among the 34 acid and bile salt-tolerant isolates, 9 (26.47%) isolates produced bacteriocins to varying degrees. All

the 34 acid and bile salt-tolerant isolates displayed sensitivity to ampicillin (10 μg/ml), tetracycline (30 μg/ml) and erythromycin (15 μg/ml), but showed resistance to kanamycin (25 μg/ml), while 17/34 of the isolates were found sensitive/resistant to streptomycin (10 μg/ml). The *in vitro* adherence to stainless steel plates of the 34 screened probiotic LAB isolates showed adhesion rates that ranged from 29.21 to 41.94%. The identification of 34 screened potential probiotic LAB strains and their genetic relatedness was performed using 16S rRNA gene sequencing and whole-genome (WGS) sequence comparisons. Hence, four isolates were identified as *Lactobacillus* species including *Lb. plantarum*, *Lb. paracasei* subsp. *tolerans*, *Lb. paracasei* and *Lb. plantarum*. The remaining 30 potential probiotic LAB strains were identified by using WGS comparisons. Thus, these isolates belonged to *Lactobacillus* and *Lactococcus* species including 22 *Lb. plantarum*, 1 *Lb. paracasei*, 1 *Lb. brevis* and 6 *Lc. lactis*. Among the 30 potential probiotic LAB strains, BAGEL predicted 1 class III bacteriocin, 7 class II bacterioicins and 3 class I bacteriocins in the genome of 9 strains. The compatibility test among the selected potential probiotic strains (*Lb. plantarum*, *Lb. paracasei* and *Lc. lactis*) showed the absence of antagonism among them. The *in vitro* antagonism testing of the potential probiotic LAB as separate pure or mixed culture against *Salmonella* showed a remarkable anti-*Salmonella* activity with 96.50 to 100% growth inhibition. The combination of strains that showed the highest growth inhibition rates against *Salmonella* Typhimurium, were used to test their *in vivo* effect on the colonization of mice by *Salmonella* Typhimurium. The survival rate of mice on day 21 after the oral challenge with *Salmonella* Typhimurium was significantly higher ($p<0.05$) in the experimental pro-typ, treated group (100% survival) when compared with the positive control group (20%

survival). The counts (colony-forming unit/ml) of *Salmonella* in feces were significantly lower ($p<0.05$) for the pro-typ group compared to the positive control group (typ). In general, this study indicates a high level of diversity in the bacterial community structure in traditional fermented foods and the LAB of the traditional fermented food samples was found to be good candidates for food industries as prospective probiotic cultures with health benefits.

Keywords/Phrases: Fermented foods, Microbial diversity, Probiotics, *Salmonella*

Acknowledgments

First and foremost, I would like to thank my supervisors Dr. Anteneh Tesfaye, Dr. Diriba Muleta and Dr. Tesfaye Sisay for their unreserved support and encouragement beginning from the very inception of this study to its completion. I am very much grateful to them for thoroughly reviewing the manuscripts and shaping the monograph to its present form through their insightful critique. I would also like to appreciate their attractive approach that was both fatherly and friendly during my study years.

I would also like to express my deepest appreciation to Prof. Per Saris, the University of Helsinki, Finland for creating the opportunity for me to visit his laboratory by covering all the expenses for my stay and basic training on molecular techniques. I would also like to extend my appreciation to Dr Timo Takala for kindly assisting and providing the reference bacteria strains during my stay at Helsinki. I also would like to thank all the staff members in the Microbiology laboratory at The University of Helsinki for sharing their valuable experiences both in the laboratory and at various social events.

I am very much grateful to Prof. Arjan Narbad, Food Innovation and Health Institute Strategic Programme, Quadram Institute Bioscience, Norwich Research Park, Norwich, the United Kingdom for accepting me to work in his laboratory and supplying with all the facilities used to identify my isolates. I am also indebted to Dr Maria Diaz for kindly introducing me to all the laboratory facilities and for her tireless guidance on the technical procedures and analyzing the sequence data. In addition, I am very much grateful to her for thoroughly reviewing the manuscripts.

I owe due thank you to the Department of Microbial, Cellular and Molecular Biology, Addis Ababa University for providing me with the admission to pursue the PhD study and Aksum University for paying my salary during this study period. My heartfelt appreciation goes also to the Ethiopian Public Health Institute (EPHI) for providing the reference strains.

Last, but not least, I would also like to thank Dr. Tesfaye Alemu, Dr. Gurja Belay, all my family and friends for their unreserved encouragement and supports during my study period.

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List of Abbreviations

AAD	Antibiotic-associated diarrhea
ATCC	American Type Culture Collection
BAGEL	Brilliantly Advanced General Electronic-structure Library
BLAST	Basic local alignment search tool
BSH	Bile salt hydrolase
CDS	Coding Sequences
CFS	Cell-free supernatant
DGGE	Denaturing gradient gel electrophoresis
EFSA	European Food Safety Authority
EPHI	Ethiopian Public Health Institute
FBD	Foodborne diseases
GI	Gastrointestinal
GRAS	Generally regarded as safe
ITS	Internal transcribed sequences
LAB	Lactic acid bacteria
MEGA7	Molecular evolutionary genetic analysis version 7
MFA	Multi-strain formula
MRS	de Man, Rogosa and Sharpe medium
NCBI	National Centre for Biotechnology Information
NGS	Next-generation sequencing
NK	Natural killer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
QIIME2	Quantitative Insights into Microbial Ecology 2
SAS	Statistical analysis software
SCFA	Short-chain fatty acids
SDS	Sodium dodecyl sulfate
TRFLP	Terminal restriction fragment length polymorphism
WGS	Whole-genome sequencing

Chapter 1 Introduction

1.1 General Introduction

Traditional fermented foods play a major role in the diet of humans across the globe, and Africa is perhaps the continent with the richest variety of fermented foods, where fermentation still plays a major role in combating food spoilage and foodborne diseases. A wide variety of raw materials are traditionally fermented in different regions of Africa, which can be classified as fermented non-alcoholic cereals, starchy root crops, animal proteins, vegetable proteins and alcoholic beverages (Franz *et al.*, 2014; Tamang and Kailasapathy, 2010). Likewise, in Ethiopia, fermented food and beverage products form an important part of the diet. Hence, these fermented food products are made from plant and animal materials in which bacteria, yeasts, and molds play a prominent role by modifying the material biochemically and nutritionally.

Foodborne diseases (FBD) pose a severe public health problem that significantly affects people's wellbeing and lead to serious socioeconomic implications (Grace, 2016). The major foodborne bacterial pathogens are *Campylobacter jejuni*, *Clostridium perfringens*, *Escherichia coli*, *Listeria monocytogenes, Salmonella* spp., and *Staphylococcus aureus* (Oliver *et al.*, 2009). These foodborne pathogens have developed multiple drug resistance and cause great economic losses in developing as well as developed countries (Lengsfeld *et al.*, 2007). The problem of foodborne disease is multifactorial and their prevention and control require multidisciplinary approaches (Sivapalasingam *et al.*, 2004).

Among the major foodborne pathogens, *Salmonella enterica* is one of the leading causes of serious illness ranging from acute gastroenteritis to systemic infections including typhoid (Castillo *et al*., 2013). Oral infection with *Salmonella* Typhimurium in mice provokes a disease similar to that caused by *Salmonella* Typhi in humans, with fever and septicemia which is lethal to the host (Santos, 2014). However, the nature and severity of the infection developed depends on many factors including the serovar involved, the virulence of the strain, the infective dose, the age and immune status of the host.

Currently, vaccination and antibiotics are used to prevent and control *Salmonella* infections. Antibiotic applications are the common clinical treatments for *Salmonella* infection, which in turn, promotes the development of resistance of *Salmonella* towards antibiotics (Berendonk *et al.*, 2015). The prolonged use of antibiotics leads to change in the intestinal commensal flora (Hammad and Shimamoto, 2010). Due to the occurrence of multidrug-resistant strains and the suboptimal efficacy of currently available vaccines, alternative intervention strategies against *Salmonella* infections are needed (Bhan *et al.*, 2005).

The consumption of a large number of probiotics together with food can fundamentally promotes the health of the consumers (Hill *et al.*, 2014). The possible mechanisms by which probiotic microbes protect against enteric pathogen infections are the production of antimicrobial substances, competition for limited resources and anti-adhesive effects (Oelschlaeger, 2010). A great number of *in vitro* and *in vivo* studies have been carried out to evaluate the effect of probiotics in the prevention and treatment of gastrointestinal infections caused by *Salmonella* (Anteneh Tesfaye *et al*., 2011; Dowarah *et al*., 2018).

The beneficial effects of probiotics are known to be genus, species, and strain-specific (Timmerman *et al.*, 2004). Currently, food-based probiotics have supposed greater significance as different food products can harbor native and beneficial probiotics and therefore can be used for both nutritional and therapeutic purposes (Meyer *et al.*, 2007).

Lactic acid bacteria (LAB) are diverse group of microorganisms consisting of Gram-positive, aerotolerant, acid-tolerant, usually nonsporulating and non-respiring rod or cocci microbes that play an important role in the process of fermentation of food by inhibiting spoilage/pathogenic bacteria by producing excellent flavor, aroma, and texture of fermented foods (Akkoc *et al.*, 2011; O'Bryan *et al.*, 2015). Some LAB are probiotics and others may be potential probiotics or just fermentation cultures that are widely distributed in nature and can be used in the food industry (Ricci *et al.*, 2019).

LAB could be isolated from many kinds of sources such as milk products, fermented foods, animal intestines or freshwater fishes, soil samples, sugar cane plants and poultry farms (Barakat *et al.*, 2011). The most common types of probiotic LAB include different *Lactobacillus* spp. (*Lb. acidophilus*, *Lb. johnsonii*, *Lb. casei*, *Lb. rhamnosus*, *Lb. gasseri* and *Lb. reuteri*) and genus *Bifidobacteria* (*Bf. bifidum*, *Bf. animalis* subsp. *lactis*, *Bf. Longum* subsp. *longum* and *Bf. longum* subsp. *infantis*) (Arthure *et al.*, 2002; Chassard *et al.*, 2011). LAB are also useful in the treatment of various diseases caused by drug-resistant pathogenic microbes (Marco *et al.*, 2017). Probiotic microbes may provide nutrients, enhance growth, produce enzymes, inhibit pathogens, and enhance immune responses (Manhar *et al.*, 2016).

Ethiopian fermented food products are well known for their unique fermentation style and can be used as a source of potentially beneficial microbes applicable as probiotics. These products are prepared at the household level, consumed as foods or beverages to support diet and nutritional intake is commonly practiced. Some studies on isolation and screening for antibacterial producing LAB and microbial diversity of traditional Ethiopian fermented foods were undertake by few workers (Negasi Akalu *et al.*, 2017; Tesfemariam Berhe *et al.*, 2019). However, there are still a few research data available on the evaluation of microbial diversity and characterization of probiotic LAB that may suppress the survival rate of human pathogen. It is, therefore, necessary to acquire a better and more accurate understanding of the microbial community probiotic potentials in traditional fermented foods derived from various regions. The availability of such information offers opportunities to study food microbes in order to understand how their potential functions can be changed or modulated with the ultimate goal of improving food quality. Most of the traditionally fermented food products of Ethiopia are consumed without further heat processing which can be considered as ideal vehicles to carry probiotic bacteria into the human gastrointestinal tract. The major objective of this study was, to examine the microbial communities associated with some of traditional fermented Ethiopia foods so as to evaluate the *in vitro* and *in vivo* probiotic properties of LAB with respect to their potential in inhibiting some foodborne pathogens. The results of this study are expected to build capacity in understanding the differences in bacterial diversity between the food samples to ultimately select optimal and appropriate beneficial bacteria for manufacturing high-quality and distinctive fermented products with health improving properties in both animals and humans.

1.2 Objectives of the Study

1.2.1 General Objective

The general objective of the present study was to assess the microbial diversity of traditionally fermented food products and to determine the *in vitro* and *in vivo* probiotic potential of lactic acid bacteria against foodborne bacterial pathogens.

1.2.2 Specific Objectives of the Study

The specific objectives of the current study were to:

i. Determine the diversity of microflora associated with some traditional fermented food products (*teff* dough, *kocho* and *ergo*).

ii. Isolate, characterize and identify LAB from selected traditionally fermented food products.

iii. Screen of potential probiotic LAB on the basis of their *in vitro* and *in vivo* characteristics.

Chapter 2 Literature Review

2.1 Traditional Food Fermentation

Traditional food processing is a very important activity in the informal sector of the economy of the world. It plays a vital role in the food supply system of the developing countries since it leads to reduction of the post-harvest loss of highly perishable crops. Traditional food processing provides a means of livelihood for a number of rural and increasingly urban women who rely on family labor and engage in commercial activity in home-based operations (Yu, 2002).

The skills of food fermentation are embedded in traditional knowledge systems among the native peoples of many areas of the world and the knowledge is maintained and propagated orally. The art of fermentation practiced by the people has continued, in spite of the technologic revolution, but has largely remained confined to the rural and tribal areas due to high cost or inaccessibility of the industry-made products in remote areas, the tastes of the people for the traditional fermented products, and their socio-cultural linkages with such products (Chelule *et al.*, 2010; Thakur and Bhalla, 2004). However, with the advent of microbiology, biochemistry, molecular biology and biochemical engineering, the art of fermentation practiced by the common man has been improved and upgraded, which has led to the rise of fermentation industries, adding quality and expanding the range of products (Thakur and Bhalla, 2004).

Food is the fundamental survival necessity for all human beings; it is usually plant or animal origin. The traditional methods of food processing were aimed at food

preservation and economy of fuel (Parkouda *et al.*, 2009; Sarkar and Nout, 2014). Food fermentation methods arose historically from the need for processing and preservation of food (Law *et al.*, 2011; Ravyts *et al.*, 2012). All over the world, a wide range of fermented food products are produced, which contribute significantly to the diets of many people (Nath *et al.*, 2016). Fermentation is an inexpensive technology, plays an important role as the major biotechnological application of food processing in many developing countries. It gives food a variety of flavors, tastes, textures, sensory attributes and nutritional and therapeutic values (Kamal-Eldin *et al.*, 2012).

The term fermented foods is used to describe a special class of food characterized by various kinds of carbohydrate breakdown in the presence of microbes (Hasan *et al.*, 2014). They are pleasant and nutritious products and have occupied an important place in culture and tradition and remained as the major component of the diet of millions of individuals. Fermented food and beverage products have emerged as not only the source of nutrition but also as functional and probiotic foods, which besides nutritional value have health effects or provide protection against diseases (Bourdichon *et al.*, 2012).

Fermented foods and beverages are a part of human nutrition since ancient times. In traditional fermented foods, the process of fermentation is spontaneous and uncontrolled. Fermented foods are associated with a unique group of microflora that enhance the nutritional quality of food such as proteins, vitamins, essential amino acids and fatty acids. In this regard, fermented food products can address the problems related to the world's balanced diet (Jeyaram, 2009). Traditional fermented food products are typically unique and vary depending on the region due to the variation in environmental

conditions, cultural and social aspects, taste preferences, availability of raw materials and new technological development (Nair, 2008).

Fermented foods contain a diverse group of microorganisms such as molds, yeasts and bacteria. In general, microorganisms come from the ingredients (of plant or animal origin), utensils, containers and the environment, and are selected through intera-and inter-microbial dynamic interaction and adaptation to the fermenting condition (Steinkraus, 1997). The interaction of diverse groups of fermentative microorganisms determine the characteristics of fermented food, such as the acidity, flavor and texture of the products and some may be health benefiting that goes beyond simple nutrition (Vogel *et al.*, 2011). They are possible to be found as the indigenous microbiota of the food or as a result of the intentional addition of microbes as starter cultures in an industrial food fermentation process (Stevens and Nabors, 2009). The beneficial organisms may have the ability to produce several compounds that have positive applications as food additives or *in situ* as a part of food fermentation processes (Longo and Sanromán, 2006).

A wide variety of fermented foods and beverages are consumed in Ethiopia. They are products of acid-alcohol type of fermentation. Most of the works hitherto addressed microbiological issues on fermentation of milk and other dairy products, fermentation of *enjerra* and *kocho* and other fermented legume and vegetable products, and beverages (Mogessie Ashenafi, 2006). Here traditional fermented products such as *ititu* from Ethiopia that ferment spontaneously and *sato* from Thailand in which starter culture is utilized in the process of traditional fermentation will be described as representative examples. Dairy products are one of the common traditional Ethiopian fermented

products made from milk of almost from all domesticated milch animals for ages. The traditional dairy fermentation process is conducted with no attempt to control the fermentation processes. Accordingly, the main fermented milk products include *ergo, ititu, ayib, kibe, arerra* and *agua*t (Mogessie Ashenafi, 2006).

Ititu is the concentrated fermented milk prepared and consumed by the Borana tribes in southern Ethiopia. This tribe prepare *ititu* during the rainy season when milk is accessible in large quantity for later consumption during the drier seasons when fresh milk supply is markedly scanty (Almaz Gonfa *et al.*, 2001). The product has good keeping quality and remains acceptable for about two months at ambient temperature (Tarik Kassaye *et al.*, 1991) to three months (Almaz Gonfa *et al.*, 2001). It is consumed as a side dish with traditional porridge or thin-baked cereal chips. It is considered as one of the special foods and served to very respected guests as well as to weaning-age children and the elderly.

During the traditional production of *ititu*, fresh milk is collected in a well smoked fermenting vessel called *gorfa* (Tarik Kassaye *et al.*, 1991). *Gorfa* is woven from fibers of selected plants into a lidded container with a capacity up to three liters. A new *gorfa* is washed with hot water, air-dried, rinsed with fresh milk and smoked for a few minutes with splinters of *Acacia nilotica* or other plants. The lid of the *gorfa* is treated with leaves of *Ocimum basilicum* for cleaning and the treatment imparts desirable flavor to the product (Almaz Gonfa *et al.*, 2001; Tarik Kassaye *et al.*, 1991). A small volume of milk (up to 300 ml) is added to the *gorfa* and is allowed to ferment naturally. When the milk coagulates, whey is removed by a wooden pipette and an additional volume of fresh milk is added. The process of removing the whey and addition of fresh milk is repeated several

times until the product is concentrated enough and is ready for consumption. Any mold growth on the surface of the curd is removed (Tarik Kassaye *et al.*, 1991).

Tarik Kassaye *et al.* (1991) studied the chemical and microbiological characteristics of *ititu* randomly collected from individual households in Borana region. The same study has indicated that *Ititu* had an average pH of 3.65, titratable acidity (as lactic acid) of 1.92%, fat of 9.05% and protein content of 7.17%. *Ititu* had increased contents of free and total amino acids when compared to fresh whole milk and was rich in amino acids such as glutamic acid, alanine, proline, leucine, and serine. In addition, Tarik Kassaye *et al.* (1991) reported that, the total bacterial count was 10^{12} CFU/g, mainly dominated by LAB. Yeast and mold counts were 10^{8} CFU/g, and coliforms were not detected. They identified the prevalent LAB like *Lb. casei* and/or *Lb. plantarum.* The counts seem exaggerated, though, and more realistic values for lactic acid bacteria of about 10^{8} CFU/g were reported by Almaz Gonfa *et al.* (2001).

Sato is a traditional alcoholic beverage of Thailand prepared from glutinous rice, a starter culture (*Loogpaeng*), and water. *Loogpaeng* as the starter culture contains different kinds of microorganisms such as fungi, yeast and bacteria. Dominant fungi are *Aspergillus*, *Mucor*, *Rhizopus*, *Amylomyces*, and *Penicillium*, while *Endomycopsis* and *Saccharomyces* are yeasts that are present in *Loogpaeng*. Along with these, acetic acid bacteria, LAB and *Bacillus* are also detected in the starter culture. During *sato* production, once the cooked glutinous rice is mixed with *Loogpaeng*, it starts to become turbid and sweet due to the saccharification caused by the amylase enzyme produced by *Amylomyces* and *Rhizopus*. After the addition of water to the sweet rice, yeast and

bacteria start to grow leading to alcoholic fermentation. Depending on the quality of *Loogpaeng*, alcohol content is about 7%−10% (v/v) in the final fermented product (Sanpamongkolchai, 2016).

2.1.1 Type of Food Fermentation

Fermentation is a form of food processing which is achieved by using microorganisms, especially lactic acid bacteria (LAB), and yeast (Mokoena *et al.*, 2016). Although it is an ancient food preservation technology, it is still part of the cultural norm usually being practiced at a local or household level among indigenous communities in developing countries (Gawai and Prajapati, 2017). In fact, fermentation is a relatively cost-effective, low-energy preservation process, which is essential to extend food shelf-life and ensures safety (Liu and Pan, 2010). Thus, it remains the major technology for important food production, even though other manufacturing technologies are available nowadays (Voidarou *et al.*, 2011). Different types of fermentation are used in food processing based on the desired end products. The four main types of fermentation widely found in food products are lactic acid fermentation, acetic acid fermentation, alkaline fermentation and alcoholic fermentation (Anal, 2019). Figure 2.1 shows the schematic representation of types of fermentation, the microorganisms involved, and the resulting end products.

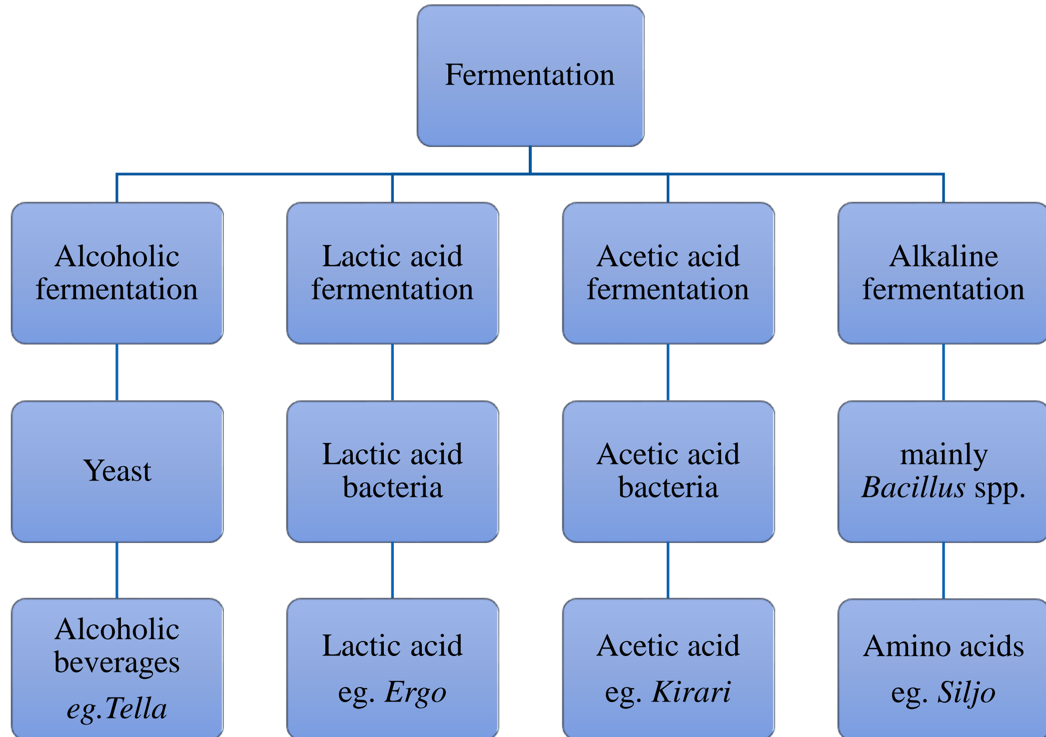

Figure 2.1: Schematic representation of the common types of fermentation, the microbes involved, and the end products

2.1.1.1. Lactic Acid Fermentation

Lactic acid fermentation results in the production of acidic foods. In lactic acid fermentation, sugars such as glucose, sucrose, and fructose are converted into cellular energy and lactic acid with the help of LAB such as *Leuconostoc*, *Lactobacillus* and *Streptococcus* bacteria. Lactic acid fermentation can further be classified into two types such as homo lactic fermentation and hetero lactic fermentation (Anal, 2019). The homo lactic fermentation is the simplest fermentation in which one molecule of glucose is ultimately converted into two molecules of lactic acid. An example of homofermentative bacteria are *Lb. delbruckii*, *Lb. bulgaricus*, *Lb acidophilus*. Homofermentative lactic fermentation is observed in the production of sour or fermented milk products, yogurt,

and bread. The hetero lactic acid fermentation differs from homo lactic fermentation in the way that one molecule of glucose is converted into one molecule of lactic acid along with a molecule of ethanol and carbon dioxide. An example of heterofermentative bacterial species are *Leuc. mesenteroide*, *Lb. brevis* and *Leuc. dextranicum*. Heterofermentative lactic fermentation is used in preserving various fruits and vegetables (Anal, 2019).

Steinkraus (1983) established the following classifications of lactic acid fermented food products: acid fermented vegetables, acid fermented bread and pancakes, acid fermented cereal gruels, acid fermented seafood/rice, and meat/rice mixtures and acid fermented milk and milk/cereal foods. Lactic acid fermentation is also important in the fermentation of traditional alcoholic and non-alcoholic beverages. Philippine *balao balao* is a lactic acid-fermented rice/ shrimp mixture prepared by packing boiled rice, raw shrimp and solar salt (about 3% w/w) in an anaerobic container and allowing the mixture to ferment over several days or weeks (Arroyo, 1977). The chitinous shell of the shrimp becomes soft and when the product is cooked, the whole shrimp can be eaten. The products are well-preserved by the low pH and anaerobiosis until the containers are opened. Another household lactic acid fermentation of considerable nutritional importance includes Egyptian *kishk* and Greek *trahanas*. These products are basically wheat/yogurt mixtures that combine the high nutritional value of wheat and milk while attaining excellent keeping qualities. Milk is fermented to yogurt and the yogurt and wheat are mixed and boiled together until the mixture is highly viscous. The mixture is allowed to cool, formed into biscuits by hand and sun-dried. *Trahanas* can be stored on the kitchen shelf for years and be used as a base for highly nutritional soups (Steinkraus, 1996).

Furthermore, an Indian *idli*, sour, steamed bread and dose, a pancake, are examples of a household lactic fermentation that could be useful around the world. Polished rice and black gram dahl in various proportions i.e. 3:1-1:3 are soaked by the housewife separately during the day. In the evening, the rice and black gram are ground in a mortar and pestle with added water to yield a thick batter. Then, the batter ferments overnight during which time *Leuc. mesenteroides* and *Strep. faecalis,* naturally present in the grains/legumes/utensils, grow rapidly producing lactic acid and carbon dioxide that leavens the product. In the morning, the batter is steamed to produce small white muffins or fried as a pancake (Steinkraus, 1996).

Similarly, traditionally Ethiopian fermented *ergo* has received extensive microbiological works and it has been found that LAB dominated all other microorganisms, followed by yeasts and then molds. According to Almaz Gonfa *et al.* (2001) report, *ergo* fermentation is carried out by lactic acid bacteria belonging to the genera, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Entrococcus* and *Streptococcus*. The same authors also reported that *Micrococcus* sp., *Coliforms* and spore formers were also present in fairly high numbers during the first 12-14 h of fermentation.

2.1.1.2. Alcoholic Fermentations

Fermentations involving the production of ethanol are also generally safe food and beverage products (Amoa-Awua *et al.*, 2006; Steinkraus, 1979). These include wines, beers, Chinese *lao-chao*, South African *kaffir*/sorghum beer and Mexican *pulque*. These are generally yeast fermentations but they also involve yeast-like molds such as *Amylomyces rouxii* and mold-like yeasts such as *Endomycopsis* and sometimes bacteria

such as *Zymomonas mobilis* (Steinkraus, 1979). The substrates include diluted honey, sugar-cane juice, palm sap, fruit juices, germinated cereal grains or hydrolyzed starch, all of which contain fermentable sugars that are rapidly converted to ethanol in natural fermentation by yeasts in the environment. Nearly equal weights of ethanol and carbon dioxide are produced and the carbon dioxide flushes out residual oxygen and maintains the fermentation anaerobic. The yeasts multiply and ferment rapidly and as a result other microorganisms, most of which are aerobic, cannot compete. The ethanol is germicidal and, as long as the fermented product remains anaerobic, the product is reasonably stable and preserved (Fleet, 2006).

The most studied African alcoholic beverages are beers produced from guinea corn (*Sorghum vulgare*). In sub-Saharan Africa, sorghum beer is known under a variety of names such as *pito* in Ghana and Nigeria and *dolo* in Burkina Faso and Togo (Konlani *et al.*, 1996; Pattison *et al.*, 1998; van der Aa Kühle *et al.*, 2001). *Burukutu* and *otika* are other sorghum-based beverages produced in Nigeria and surrounding countries (Iwuoha and Eke, 1996). The brewing of indigenous sorghum beer involves malting, souring, boiling, mashing, straining and alcoholic fermentation (Pattison *et al.*, 1998). The microbiology of *pito* and *dolo* has been investigated (van der Aa Kühle *et al.*, 2001). Samples of dried yeasts harvested from previous brews and used as inoculums in beer fermentation were found to contain $0.2\text{-}7.6 \times 10^7$ cells/g (van der Aa Kühle *et al.*, 2001). When the yeasts were identified, 99% of the isolates showed macro and micro morphological characteristics typical of *S. cerevisiae* whereas 1% was identified as *Candida kefyr*. The dominance of *S. cerevisiae* in the fermentation of *pito* and other indigenous fermented sorghum beers seems to be a general observation. However, the

occurrence of other yeast species seems to vary. *S. cerevisiae* was found in a number of Nigerian cereal-based beverages including *pito* and *burukutu* (Sanni and Lönner, 1993).

In Asia, there are at least two additional ways of fermenting starchy rice to alcoholic foods. The first is the use of a mold such as *Amylomyces rouxii* which produces amylases converting starch to sugars and yeast such as *Endomycopsis jibuliger* which converts the glucose/maltose to ethanol. The sweet-sour/alcoholic product of rice fermentation is called *tape ketan* in Indonesia and is consumed as a dessert. When cassava is used as the substrate, the product is called *tape ketella* (Merican and Quee-Lan, 2004; Steinkraus, 1996). Another method is the Japanese *koji* process used to ferment rice to rice wine (*sake*) (Yoshizawa and Ishikawa, 2004). In this process, boiled rice is overgrown with an amylolytic mold *Aspergillus oryzae* for about 3 days at 30°C. The mold-covered rice called *koji* is then inoculated with a culture of the yeast *S. cerevisiae* and water is added. Saccharification by the mold amylases and alcoholic fermentation by the yeast proceed simultaneously. The result of slow fermentation is high yeast populations and ethanol contents as high as 23% v/v.

In Ethiopia, very popular traditional fermented alcoholic drinks include *tella* (Berhanu Abegaz, 2014), *tej* (Bekele Bahiru *et al.*, 2001), *areki* (Tadele Yohannes *et al*., 2013), *borde* (Kebede Abegaz, 2007) and *shamita* (Ketema Bacha *et al.*, 1999). *Tej* fermentation, like other traditional beverages of Ethiopia, is a natural fermentation and no starter culture or other modern techniques are used. *Tej* is a mead that is prepared from honey, water, and leaves of *gesho* (*Rhamnus prinoides*) (Bekele Bahiru *et al.*, 2001). Mix one-part honey to three parts water, put in some stems and branches of *gesho* and let it

ferment for 5 to 6 weeks, removing the *gesho* after 2 weeks. *Tej* fermentation depends upon the microorganisms present in the environment. Thus, to determine the major source of the yeast cells in *tej* attention was given to honey and *gesho*. The dominant yeast, *S. cerevisiae*, counts ranged between 10^2 to 10^3 CFU/g in *gesho* samples while 0 to 10^2 in honey samples. *Gesho* was considered the major source of the dominant yeast in *tej* because it contained a greater number of the yeast than honey

2.1.1.3. Acetic Acid /Vinegar Fermentation

Acetic acid bacteria are also commonly found in a wide range of fermented foods and beverages. They are ubiquitous, aerobic, Gram-negative bacteria belonging to the Acetobacteraceae, and the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter* and *Komagataeibacter* constitute the common acetic acid bacteria found in food and beverage fermentation such as *cocoa*, *milk kefir*, *water kefir*, *kombucha* and *acidic beers* (Pothakos *et al.*, 2016). This type of fermentation results in the production of acetic acid foods or condiments that are generally considered as safe, as acetic acid is either bacteriostatic or bactericidal, depending upon the concentration employed (Steinkraus, 1997). When the products of alcoholic fermentation are not kept anaerobic, bacteria belonging to the genus *Acetobacter* present in the environment oxidize portions of the ethanol to acetic acid/vinegar (Conner and Allgeier, 1976; Steinkraus, 1983; Steinkraus, 1995; Steinkraus, 1997). Vinegar is a highly acceptable condiment used in pickling and preserving cucumbers and other vegetables. It is characterized by significant acetic acid content, made from various alcoholic products using acetic acid bacteria. Vinegar is not a food of

major importance to the diet but plays an important role as a highly effective food preservative and flavoring agent of biological origin (Steinkraus, 1997).

According to Samuel Sahle and Berhanu Abegaz (1991) report over 2 million hectoliters of *tella* are thought to be produced annually in households and *tella* vending houses in Addis Ababa. The fermenting organisms were composed of *S. cerevisiae* and *Lb. pastorianumi*. The yeasts dominated the fermenting flora. An increase in alcohol content was accompanied by yeast growth and a decrease in reducing sugars and total carbohydrates. The pH and ethanol content is in the range of 4.5-4.8 and 2.8-5.0% (v/v) respectively, when *tella* is considered to be the most suitable for consumption. However, the same author revealed that at the end of fermentation, *tella* becomes too sour to consume due to the growth of *Acetobacter* spp. which converts ethanol to acetic acid under aerobic conditions. Furthermore, some fermented foods produced by acetic acid bacteria are *apple cider* and *wine vinegars* in the West; *palm wine vinegars* in Africa and the Far East, *coconut water vinegar* in the Philippines; *tea fungus*/ *Kombucha* in Europe, Manchuria, Indonesia, Japan (Steinkraus, 2002).

2..1.1.4 Alkaline Fermentations

Alkaline fermentation is a process in which the pH of the substrate increases to alkaline values as high as 9 (Joshi, 2016) due to the enzymatic hydrolysis of proteins from the raw material into peptides, amino acids and ammonia. In developing countries, the traditional diets of the majority of people depend largely on starchy staples but poor in other nutrients (Achi, 2005; Dakwa *et al.*, 2005). The microorganisms involved in alkaline fermentation are *Bacillus subtilis* and related bacilli. Fermented food condiments form a

significant part of the diets of many people in developing countries (Parkouda *et al.*, 2010). Meaty/savor flavored amino acid/peptide sauces and pastes, fish sauces and pastes, made by fermentation of small fish and shrimp, using principally their proteolytic gut enzymes are also included in this type of fermentation (Joshi, 2016).

Moreover, the Malaysians ferment locust beans by a similar alkaline process to yield garlic-flavored products. The Japanese ferment soybeans with *B. subtitles* after soaking and cooking to yield a protein-rich food called *natto*. The Thais ferment soybeans by a similar process to produce a product called *thua-nao* consumed in Northern Thailand as a substitute for fish sauce. The Indians also ferment soybeans by similar processes to produce *kinema*. These alkaline fermentations involving bacilli-fermenting protein-rich beans and seeds are of considerable importance in widely separated parts of the world. They are all household fermentations but Japanese *natto* has been commercialized (Steinkraus, 1991).

2.1.2 Role of Food Fermentation

Fermentation has had a deep impact on the history of food, eating habits and cultural exchange of food. Although fermentation has a number of functional aspects, it primarily evolved as a process to preserve food safely. Food fermentation provides several advantages with respect to the improvement in the quality and nutritional value; it makes the foods more digestible, it improves the sensory attributes, it synthesizes vitamins, it destroys undesirable flavors, and has ability to replenish intestinal microflora, it can also help to reduce the volume of material to be transported and reduce the energy required for the subsequent cooking of preserved products. It can improve protein quality and the

bioavailability of micronutrients and can reduce toxic and ant-nutritional factors (Ebner *et al.*, 2014; Simango, 1997; Steinkraus, 2002).

2.1.2.1. Nutritional Quality

A number of foods especially cereals are poor in nutritional value, and because they are generally cheap, they constitute the main staple diet of the low-income populations (Achi and Asamudo, 2018). The acidic nature of the fermented products enhances the activity of microbial enzymes at a temperature range of 22-25ºC (Mokoena *et al.*, 2005). The enzymes, which include amylases, proteases, phytases and lipases, modify the primary food products through hydrolysis of polysaccharides, proteins, phytates and lipids respectively (Chelule *et al.*, 2010; Mokoena *et al.*, 2005; Yang *et al.*, 2019). Thus, in addition to enhancing the activity of enzymes, LAB fermentation also reduces the levels of anti-nutrients such as phytic acid and tannins in fermented foods leading to increased bioavailability of minerals such as iron, protein and simple sugars (Chelule *et al.*, 2010). The amount of vitamins is also increased in fermented food products (Fardet and Rock, 2018).

Generally, fermented foods can be more nutritious than their unfermented counterparts (Oladeji *et al.*, 2018). First, microorganisms not only are catabolic, breaking down more complex compounds, but they also are anabolic and synthesize several complex vitamins and other growth factors. The second important way in which fermented foods can have improved nutritionally has to do with the liberation of nutrients locked into plant structures and cells by indigestible materials. This is especially true in the case of certain grains and seeds. The third mechanism by which fermentation can enhance nutritional

value, especially of plant materials, involves enzymatic splitting of cellulose, hemicellulose and related polymers that are not digestible by humans into simpler sugars and sugar derivatives (Hasan *et al.*, 2014; Oladeji *et al.*, 2018). Cellulosic materials in fermented foods can be nutritionally improved for humans by the action of microbial enzymes (Hasan *et al.*, 2014).

2.1.2.2 Enhancement of Sensory Quality

Fermentation improves the diversity of flavors, aromas and textures in food substrates thereby adds new sensory properties to the diet and makes the food more palatable and, ultimately, more popular than the unfermented food (Blandino *et al.*, 2003; Caplice and Fitzgerald, 1999; Chelule *et al.*, 2010). These organoleptic properties make fermented food products more popular than the unfermented ones in terms of consumer acceptance (Blandino *et al.*, 2003). The agents responsible for these properties have been described (Ramaite and Cloete, 2006). The addition of other ingredients like citric acid in lemon juice, to imitate the low pH of fermented products failed to give the same desired results. Furthermore, food acidification does not result in the improvement of food texture and quality. Thus, fermentation is unique in that it modifies the unfermented food in diverse ways, resulting in new sensory properties in the fermented product (De Vuyst and Leroy, 2007). However, not all bacteria and molds are beneficial in enhancing food flavor. In some instances, they may cause food spoilage since their enzymes may lead to generate fermentation digests that have offensive odors or flavors, making food to be entirely unpalatable. Alkaline fermentation is known to play an important role in making otherwise inedible foods edible, enhancing flavor and nutritional values and bringing

diversity to the kinds of foods and beverages available (Chukeatirote *et al.*, 2010; Parkouda *et al.*, 2009; Steinkraus, 1995; Wang and Fung, 1996).

2.1.2.3 Bio-preservation of Foods

Fermentation plays a prominent role in preserving food by producing antimicrobial compounds to prevent pathogenic organisms through the activities of microorganisms or their enzymes. The preservative effect of fermented food products is also due to several antimicrobial compounds synthesized during fermentation (Santos *et al.*, 2018). In lactic acid fermentation, the inhibitory compounds produced by lactic acid bacteria against other microbes include hydrogen peroxide, reutrin, diacetyl, reutericyclin and bacteriocins (Chelule *et al.*, 2010; Ouwehand and Vesterlund, 2004; Santos *et al.*, 2018). These bacteria also produce fungal inhibitory metabolites including organic acids that are also a hurdle for non-acid-tolerant bacteria (Chelule *et al.*, 2010; Mensah, 1997). Carbon dioxide is produced from heterolactic fermentation can directly create an anaerobic environment that is toxic to some aerobic food microorganisms through its action on cell membranes and its ability to reduce both internal and external pH (De Vuyst and Vandamme, 1994; Eklund, 1984). In general, many of the fermented foods have good keeping qualities and can be kept without refrigeration for more time than the fresh unfermented foods (Jespersen, 2003; Joshi, 2006a; Pandey *et al.*, 2000). Fermentation increases the shelf-life and decreases the need for refrigeration or other method of food preservation (Aidoo, 2011; Cooke *et al.*, 1987).

2.1.2.4 Health Benefits

LAB fermentation prevents diarrheal diseases as they modify the composition of intestinal microorganisms, constipation, and abdominal cramps, acting as deterrents for pathogenic enteric bacteria (Chelule *et al.*, 2010). Mostly species of LAB used for the production of fermented foods are those having generally recognized as safe (GRAS) status, thus eliminating any health risk (Ghosh *et al.*, 2016; Hansen, 2002; Nout, 2001). These bacteria are normal residents of the complex ecosystem of the gastrointestinal tract (GIT) (Tamang, 2003). The probiotic effects and the reduced level of pathogenic bacteria documented in fermented foods are of special significance when it comes to the developing countries, where fermented foods has been shown to have potentials to reduce the severity, duration, and morbidity from diarrhea (Jespersen, 2003; Mensah *et al.*, 1990). On another note, a large cohort studies in the Netherlands and Sweden have examined the effects of regular consumption of fermented dairy products on the risk of bladder cancer (Keszei *et al.*, 2009) and cardiovascular disease (Sonestedt *et al.*, 2011). In general, both the microbes and fermented products have various functionalities in maintaining human health (Ghosh *et al.*, 2016).

2.1.2.5 Detoxification and Antimicrobial Components

Foods and feeds are often contaminated with a number of toxins, either naturally or through contamination with microbes such as molds, bacteria, and viruses (Chelule *et al.*, 2010). Certain molds often produce secondary toxic metabolites called mycotoxins. These include fumonisins, ocratoxin A, zearalenone and aflatoxins (Kabak and Dobson, 2017). Several methods are available for degrading toxins from contaminated food, for

example, using alkaline ammonia treatment to remove mycotoxins from food. However, these methods are harsh to food as they involve the use of chemicals which are potentially harmful to health or may reduce the nutritional value of food. Cooking food does not remove mycotoxins either, as most of them are heat-stable. Fermentation is known to play an important role in detoxifying these compounds and other contaminated foods (Olukoya *et al.*, 1994; Ross *et al.*, 2002). Using lactic acid bacteria fermentation for detoxification is more advantageous in that it is a milder method that preserves the nutritive value and flavor of decontaminated food (Manubolu *et al.*, 2018). The detoxifying effect is believed to be through a toxin binding effect (Lili *et al.*, 2017). Other authors allude to the possibility of an enzymatic interaction, although this was not thoroughly investigated (Zinedine *et al.*, 2005).

As in the case of mycotoxin detoxification, LAB fermentation contribute acidity to cassava fermentation and the endogenous linemarase (cyanogen glucosidase) enzyme from cassava detoxify cassava toxins (cyanogens) (Caplice and Fitzgerald, 1999). In addition to cyanogen detoxification, cassava fermentation contributes to the preservation and improvement of flavor and aroma of cassava ferment (Holzapfel, 1997). Although cooking has been used as a method of cyanogens detoxification, it has a number of problems as it leaves residual cyanogens in processed cassava, which exist as glucoside, cyanohydrin or free cyanide, which are equally toxic as their parent compounds in uncooked food (Chelule *et al.*, 2010).

2.1.2.6 Reduction of Cooking Times

Economy of fuel necessities is very important in the developing world where housewives may spend hours every day collecting enough leaves, twigs, wood and dried dung (Lynch, 2002). Fermented foods generally require little, if any, heat in their fermentation and can be consumed without cooking. Examples are pickled vegetables, *sauerkraut, kimchi*. Indonesian *tempe* fermentation converts soybeans that would require as much as 5 to 6 h cooking to a product that can be cooked in soup with 5 to 10 min boiling (Steinkraus, 1983). Therefore, the fermentation process is less energy consuming and requires less costly equipment and, thus, is a cheap and efficient means of preserving perishable raw materials (Egounlety, 2002).

2.1.3 Classification of Fermented Foods and Beverages

According to Campbell-Platt (1987) report, there are around 3500 global fermented foods and beverages and divided them into about 250 groups. There might be more than 5000 varieties of common and uncommon fermented foods and alcoholic beverages being consumed in the world today by billions of people, as staple and other food components (Tamang and Kailasapathy, 2010). Global fermented foods are classified into nine major groups on the basis of substrates used from plant/animal sources: (1) fermented cereals, (2) fermented vegetables and bamboo shoots, (3) fermented legumes, (4) fermented roots/tubers, (5) fermented milk products, (6) fermented and preserved meat products, (7) fermented, dried and smoked fish products, (8) miscellaneous fermented products and (9) alcoholic beverages (Steinkraus, 1997; Tamang and Kailasapathy, 2010).

Dirar (1993) stated that the Sudanese classify their fermented foods, not on the basis of microorganisms or commodity but on a functional basis: (1) *Kissar* (staples)-porridges and breads such as *aceda* and *kissra*; (2) *Milhat* (sauces and relishes for the staples); (3) *Marayiss* (30 types of opaque beer, clear beer, date wines and meads and other alcoholic drinks); and, (4) *Akil-munasabat* (food for special occasions). Likewise, in Ethiopia, the fermented food and beverage products can be classified into five groups according to the substrates or raw materials employed. These include; (1) fermented plant products (*injerra*, and *kocho*), (2) fermented dairy products (*ergo, kibe, arera*, *ayib, ititu* and *dhanaa*), (3) fermented beverages (*tella, tej, borde, shamata, keribo* and *korefe*), (4) fermented meat (*sausage*) and (5) fermented condiments (*awaze, siljo* and *datta*) (Berhanu Abegaz, 1987; Ketema Bacha *et al.*, 1998; Mogessie Ashenafi, 2002).

2.1.4 Diversity of Microorganisms in Fermented Foods

The natural fermentation takes place with the community of microbes such as molds, bacteria, and yeasts (Antony and Chandra, 1997). Thus, fermentation products are based on the microbes involved in the biochemical changes. LAB are widely present in many fermented foods (Stiles and Holzapfel, 1997; Tamang and Kailasapathy, 2010). The major genera of the LAB such as *Alkalibacterium, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus and Weissella* (Axelsson *et al.*, 2012; Holzapfel and Wood, 2014; Salminen and Von Wright, 2004) have been isolated from various globally fermented foods and beverages. *Bacillus* is present in alkaline fermented foods of Asia and Africa (Parkouda *et al.*, 2009; Tamang, 2015b). Species of *Bacillus* that are present,

mostly in legume-based fermented foods, are *B. amyloliquefaciens, B. circulans, B. coagulans, B. firmus*, *B. licheniformis, B. megaterium, B. pumilus, B. subtilis* and *B. thuringiensis* (Kiers *et al.*, 2000; Kubo *et al.*, 2011), while strains of *B. cereus* have been isolated from the fermentation of *Prosopis africana* seeds for the production of *okpehe* in Nigeria (Oguntoyinbo *et al.*, 2007). Some strains of *B. subtilis* produce λ-polyglutamic acid (PGA) which is an amino acid polymer commonly present in Asian fermented soybean foods, giving the characteristic of a sticky texture to the product (Nishito *et al.*, 2010; Urushibata *et al.*, 2002).

The association of several species of *Micrococcus* (members of the *Actinobacteria*), and *Staphylococcus* (belonging to the *Firmicutes*) has been reported for the fermentation of fermented milk products, fermented sausages, meat and fish products (Coton *et al.*, 2010; Martín *et al.*, 2006). Species of *Bifidobacterium, Brachybacterium, Brevibacterium,* and *Propionibacterium* are isolated from cheese, and species of *Arthrobacter* from fermented meat products (Bourdichon *et al.*, 2012). *Enterobacter cloacae*, *Klebsiella pneumoniae, Klebsiella pneumonia* subsp. *ozaenae, Haloanaerobium, Halobacterium, Halococcus*, *Propionibacterium, Pseudomonas,* etc. are also present in many global fermented foods (Tamang and Samuel, 2010b).

Alcohol fermentation contributes to the production of ethanol. Yeasts are the predominant organisms in the production of wines, beers, vodka, whiskey, brandy and bread (Anal, 2019). Genera of yeasts reported for fermented foods, alcoholic beverages and non-food mixed amylolytic starters are mostly *Brettanomyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Dekkera*, *Galactomyces*, *Hyphopichia*, *Issatchenkia*,

Kazachstania, *Kluyveromyces*, *Rhodosporidium*, *Saccharomyces*, *Saccharomycodes*, *Saccharomycopsis*, *Schizosaccharomyces*, *Sporobolomyces*, *Torulaspora*, *Torulopsis*, *Trichosporon*, *Yarrowia* and *Zygosaccharomyces* (Tamang and Fleet, 2009).

The major role of filamentous molds in fermented foods and alcoholic beverages is the production of enzymes and the degradation of anti-nutritive factors (Aidoo and Nout, 2010). Species of *Actinomucor, Amylomyces, Aspergillus, Monascus, Mucor, Neurospora, Parcilomyces, Penicillium, Rhizopus* and *Ustilago* are reported for many fermented foods, Asian non-food amylolytic starters and alcoholic beverages (Chen *et al.*, 2014; Nout and Aidoo, 2002). Some of the common microbes associated with different traditional fermented food and beverage products are shown in Table 2.1.

Table 2.1: Microbes associated with the fermentation of different traditional fermented food and beverage products

Fermented product	Substrate	Country	Microorganisms involved	References
Ogi	Maize, millet	Nigeria	*Lb. plantarum, Lb. fermentum, S. cerevisiae, Candida krusei, Corynebacterium spp., Acetobacter spp.*	(Kostinek *et al.*, 2005; Kuye and Sanni, 1999)
Togwa	Cassava, sorghum	Tanzania	*Lb. spp., Pediococcus pentosaceus, Weissella confusa, Issatchenkia orientalis, S. cerevisiae, Candida pelliculosa*	(Mugula *et al.*, 2003)
Amasi	Milk	South Africa	*Lb. delbrueckii* and *Streptococcus spp.*	(Osvik *et al.*, 2013; Oyewole, 1997)
Bushera	Sorghum	Uganda	*Lb. spp., Streptococcus spp., Leuconostoc spp., Pediococcus spp., Weissella spp.*	(Muyanja *et al.*, 2003)
Chibuku	Sorghum	Zimbabwe	*Lactobacillus spp., Saccharomyces cerevisiae*	(Gadaga *et al.*, 1999)
Ergo	Milk	Ethiopia	*Lactobacillus, Lactococcus, Streptococcus, Leuconostoc*	(Mogessie Ashenafi, 2002)
Labanrayeb	Milk	Egypt	*Lb. casei, Lb. plantarum, Lb. brevis, Lact. lactis, Leuconostoc spp*.	(Bernardeau *et al.*, 2006)
Airag	Mare, camel milk	Mongolia	*Lb. helveticus, Lb. kefiranofaciens, Bifidobacterium mongoliense*, *Kluyveromyces marxianus*	(Bernardeau *et al.*, 2006)
Kefir	Goat, sheep, cow milk	Russia	*Lb. brevis, Lb. caucasicus, Strep. thermophilus, Lb. bulgaricus, Lb. plantarum, Lb. casei, Lb. brevis, Tor. holmii, Tor. Delbruechii*	(Bernardeau *et al.*, 2006)
Boza	Cereals	Bulgaria	*Lb.* spp., *Lc.* spp., *Pediococcus* spp., *Leuconostoc* spp.,	(Blandino *et al.*, 2003)

			S.cerevisiae	
Busa	Maize, sorghum, millet	Kenya	*S. cerevisiae*, *Schizosacchromyces pombe*, *Lb. plantarum, Lb. helveticus, Lb. salivarius, Lb. casei, Lb. brevis, Lb. buchneri, Leuc. mesenteroides, Ped. Damnosus*	(Wood, 2012)
Hussuwa	Sorghum	Sudan	*Lb. fermentum, Ped. acidilactici, Ped. pentosaceus*, Yeasts	(Yousif *et al.*, 2010)
Jalebi	Wheat flour	India, Nepal, Pakistan	*S. Bayanus, Lb. fermentum, Lb. buchneri, Lc. lactis, Ent. faecalis, S. Cerevisiae*	(Steinkraus, 1995)
Burong mustala	Mustard	Philippines	*Lb. brevis, Ped. Cerevisiae*	(Rhee *et al.*, 2011)
Cucumbers (fermented)	Cucumber	Europe, USA, Canada	*Leuc. mesenteroides*, *Ped. cerevisiae, Ped. acidilactici, Lb. plantarum, Lb. brevis*	(Lindgren and Dobrogosz, 1990)
Oiji	Cucumber	Korea	*Leuc. mesenteroides, Lb. brevis, Lb. plantarum, Ped. Cerevisiae*	(Alexandraki *et al.*, 2013)
Naw-mai-dong	Bamboo shoots	Thailand	*Leuc. mesenteroides, Ped. cerevisiae, Lb. plantarum, Lb. brevis, Lb. fermentum, Lb. buchneri*	(Bhithakpol *et al.*, 1995)
Douchi	Soybean	China, Taiwan	*B. amyloliquefaciens, B. subtilis, Asp. Oryzae*	(Wang *et al.*, 2006)
Chikwangue	Cassava	Zaire	*Corynebacterium, Bacillus, Lactobacillus,Micrococcus, Pseudomonas, Acinetobacter, Moraxella*	(Adewumi *et al.*, 2013)
Tapé	Cassava	Indonesia	*Streptococcus* sp., *Rhizopus* sp., *Saccharomycopsis fibuligera*	(Ohba *et al.*, 1989)
Androlla	Pork	Spain	*Lb. sake, Lb. curvatus, Lb. plantarum*	(Fontán *et al.*, 2007)

2.2. Lactic Acid Bacteria

Lactic acid bacteria are Gram-positive bacteria that are grouped together by sharing morphological, metabolic and physiological characteristics. They are nonsporulating rods or cocci, acid-tolerant, anaerobic or microaerophilic and catalase-negative. Additionally, most of the LAB members are capable of converting lactose and other sugars to lactic acid (Chowdhury *et al.*, 2012). Although they are mesophilic, some can grow below 5^oC and others at temperatures as high as 45^oC. Some LAB can grow in acidic pH (3.2) and others in alkaline pH (9.6), with most growing in the pH range 4.0-4.5 (Jay, 2000). Salt tolerance (6.5% NaCl) may also be used to distinguish LAB, although variable reactions can be found among them (Mundt, 1986). However, extreme salt tolerance (18% NaCl) is confined to the genus *Tetragenococcus.*

In general, LAB are found in two distinct phyla, namely Firmicutes and Actinobacteria. Within the Firmicutes, lactic acid bacteria belong to the order Lactobacillales and include the following genera: *Aerococcus, Alloiococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Symbiobacterium, Tetragenococcus, Vagococcus and Weissella*, which are all, have low guanine-cytosine content organisms (31–49 %). And also LAB in Actinobacteria phylum only includes species of *Bifidobacterium* genus, which are high G+C content organisms (55–67 %) (Horvath *et al.*, 2009).

Lactic acid bacteria obtain their energy by substrate-level phosphorylation because they do not possess a functional respiratory system. They have used two different metabolic

pathways: the homo and heterofermentative pathway (Salminen and Von Wright, 2004). The homofermentative pathway follows glycolysis (Embden–Meyerhof–Parnas pathway) and produces virtually only lactic acid as their end product. While the heterofermentative LAB uses the pentose phosphoketolase pathway and produces lactic acid, significant amounts of CO_2 and ethanol or acetate (Axelsson, 2004). Therefore, the mode of fermentation in combination with physiological characteristics such as temperature ranges for growth and sugar utilization patterns were used as classification criteria to allocate LAB to different genera and species (Salminen and Von Wright, 2004).

LAB are found in diverse habitats wherever carbohydrate substrates are available. These include food and feed (dairy products, grain products, meat and fish products, beer, wine, fruits and fruit juice, pickled vegetables, mash, sauerkraut, silage and sourdough), water, soil, sewage and the oral (mucous membranes), respiratory, gastrointestinal and genital tracts of humans and animals (Horvath *et al.*, 2009).

2.2.1 Phylogenetic Classification of LAB

Earlier characterization of LAB relied on simple microbiological methods based on physical and phenotypic characteristics (Axelsson and Ahrné, 2000). Their major problem is reliance on phenotypic expression which is bound to be influenced by factors such as genotype and environment. They have also many weaknesses like limited accuracy, discriminating power, reproducibility and are tedious since several tests are required for characterization even at the genus level. However, these methods still remain important for preliminary classification and understanding of the strain properties. The drawbacks of these methods have led to the emergence of DNA based molecular methods

for the characterization of LAB. Thus, molecular biology methods have greatly increased the quality, reproducibility, accuracy, and efficiency of identification of microorganisms and discrimination between closely related species (Singh *et al.*, 2009).

Today, there is a means to examine, in detail, macromolecules of the cell, believed to be more accurate in defining relationships and phylogenetic positions. These are, of course, the nucleic acids. Close relations (at species and subspecies level) can be determined with DNA-DNA homology studies (Klaenhammer, 1993). For determining phylogenetic positions of species and genera, ribosomal RNA (rRNA) is more suitable, since the sequence contains both well-conserved and less conserved regions. It is now possible to determine the sequence of long stretch of rRNA (~1500 bases of 16S rRNA) from bacteria (Pang *et al.*, 2011) comparisons of these sequences are currently the most powerful and accurate technique for determining phylogenetic relationships of microorganisms (Horvath *et al.*, 2009). In addition, rRNA sequencing is becoming an important aid in the classification of lactic acid bacteria, as exemplified by the descriptions of new genera (De Klerk, 1967; Dower *et al.*, 1988).

The use of 16S rRNA gene sequences has been by far the most common housekeeping genetic marker to study bacterial phylogeny and taxonomy for a number of reasons such as (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed; and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Janda and Abbott, 2007). Apart from this, additional data are required to propose a new bacterial taxon that includes the source of isolation; phenotype variations such as optimum growth conditions, gram

staining, colony morphology, motility, spore-forming capacity; biochemical profiles including oxidase and catalase testing and carbohydrate metabolizing ability. Antibiotic susceptibility of the isolates is also important in some cases (Varsha, 2015). Apart from 16S rRNA gene, sequences of 23S rRNA gene, 16S-23S rRNA gene internal transcribed sequences (ITS), housekeeping genes such as rpoB gene encoding the β- subunit of DNA gyrase and groELgene encoding the heat shock protein are also important when characterizing a new bacterium (Drancourt and Raoult, 2005).

Genome analysis and comparison provide insights into the metabolic potential, characteristics and evolution of LAB (Pfeiler and Klaenhammer, 2007; Siezen *et al.*, 2004). The falling costs and less time for whole-genome sequencing (WGS) have already resulted in the application of this method in diagnostic microbiology and surveillance (Grad *et al.*, 2013; Rasko *et al.*, 2011). Whole-genome sequences are also useful in functional genomics studies for mapping the RNA sequence reads (Sorek and Cossart, 2010). WGS can be considered as the ultimate source of information and complete closed genome sequences as permanent, valuable scientific resources (Fraser *et al.*, 2002). In genomic studies of spoilage bacteria, identifying metabolic pathways associated with spoilage reactions is essential, as is functional analyses utilizing cloning techniques, transcriptomics and metabolomics (Remenant *et al.*, 2015).

Comparative genomics of fully-sequenced LAB genomes have revealed that the genomes of these organisms are relatively small, between 1.8 to 3.3 Mb, with the number of genes in the range of 1200 to 3000 (Makarova and Koonin, 2007; Pfeiler and Klaenhammer, 2007). Characteristic for the divergence of Lactobacillales from their ancestor *Bacilli* was

substantial loss of genes, including genes for biosynthetic enzymes and for sporulation, due to adaptation to more nutrient-rich environments (Makarova and Koonin, 2007; Pfeiler and Klaenhammer, 2007). The majority of the genome sequences used in the comparative genomics studies represented the genus *Lactobacillus*, whereas only one *Leuconostoc* and a few *Lactococcus* genomes were included (Pfeiler and Klaenhammer, 2007). Within the genus *Lactococcus*, whole-genome sequences are only available for strains of the species *Lc. lactis* and recently, *Lc. garvieae*, whereas the genome of *Lc. piscium* is still lacking (Rahkila, 2015). Within the genus *Leuconostoc* species relevant in the meat environment, the genomes of *Leuc. gasicomitatum* and *Leuc. gelidum* have been published (Jung *et al.*, 2012). The genome of *Leuc. gasicomitatum* possessed genes required for the utilization of ribose, external nucleotides, nucleosides and nucleobases, which all are abundant in meat. The genes associated with buttery off-odor, the greening of meat and slime formation were recognized, as well as genes associated with platelet binding and collagen adhesion (Johansson *et al.*, 2011). In general, the growing number of fully-sequenced genomes of LAB will provide a basis for more comprehensive genomic studies in the future.

In recent years, the development of next-generation sequencing (NGS) techniques (Nowrousian, 2010) allowing large-scale analysis of microbial communities resulted in novel applications, such as metagenomics (Xu, 2006). Metagenomics is the study of genomic material obtained directly from the environment instead of from culture (Planý *et al.*, 2016). The development of NGS technologies, such as pyrosequencing of 16S rRNA genes, provided abundant sampling depth compared to traditional approaches, such as DGGE, TRFLP, or 16S rRNA gene clone libraries. However, the intrinsic error rate of

pyrosequencing may result in the overestimation of rare phylotypes (Simon and Daniel, 2011). To date, metagenomic analysis has been used in various environments, and more than 210 different metagenomes have been sequenced from a large variety of environments, such as soil, global oceans, the human gut, and feces (Goll *et al.*, 2010). The application of metagenomics was constantly expanded in recent years, such as (a) bioprospecting from metagenomic libraries, (b) mining of metagenomes from extreme environments, (c) assessment of taxonomic and functional diversity of microbial communities (Simon and Daniel, 2011).

2.3 Probiotics and their Properties

Elie Metchnikoff, a Russian scientist working at the Pasteur Institute in Paris is credited with calling attention to the health benefits of yogurt. His hypothesis was that the LAB in the yogurt counteracts the harmful putrefying bacteria in the intestines (Hughes and Hoover, 1991). At this time, Henry Tissier isolated *Bifidobacteria* from the stools of breast-fed infants and found that they were a predominant component of the intestinal flora in humans (Ishibashi, 1993). Tissier recommended the administration of *Bifidobacteria* to infants suffering from diarrhea, believing that the *Bifidobacteria* would displace putrefactive bacteria responsible for gastric upsets while re-establishing themselves as the dominant intestinal microorganisms (O'sullivan *et al.*, 1992).

The term probiotic meaning “for life” and it is currently used to name bacteria associated with beneficial effects for humans and animals (Suvarna and Boby, 2005). But the definition of probiotics has evolved over time simultaneously with the increasing interest in the use of viable bacterial supplements and in relation to the progress made in

understanding their mechanisms of action. However, the term ‘probiotics’ was first introduced by Lilley and Stillwell (1965) to describe, "growth-promoting factors" produced by microorganisms. And then in 1974 Parker proposed that probiotics are 'organisms and substances which contribute to intestinal microbial balance' (Fioramonti *et al.*, 2003). In more modern definition Salminen *et al.* (1998) defined probiotics as the 'food which contains live bacteria beneficial to health', whereas Charteris *et al.* (1997) defined probiotics as 'microorganisms which, when ingested, may have a positive effect on the prevention and treatment of a specific pathologic condition'. Since probiotics have been found to be effective in the treatment of some gastrointestinal diseases (Marteau *et al.*, 2001), they can be considered to be therapeutic agents. The universal definition of probiotic was established by the World Health Organization (WHO) and Food and Agriculture Organization of the United States (FAO). These two organizations defined probiotics as ‘live microorganisms which when administered in adequate amounts, have a beneficial effect on the health of the host organism (Corcionivoschi *et al.*, 2010).

2.4 Probiotic Microorganisms

The most common types of microorganisms used as probiotics are LAB and *Bifidobacteria*, although other bacteria and certain yeasts are also used (Didari *et al.*, 2014). LAB are associated with habitats that are rich in nutrients, such as various food products and plant materials. They can be found in soil, water, manure, sewage and silage and can ferment or spoil food. Particular LAB are inhabitants of the human oral cavity, the intestinal tract, and the vagina, and may have a beneficial influence on these human ecosystems. They may therefore also be candidates for application as probiotics

(Holzapfel *et al.*, 2001; Reuter, 1965; Reuter, 2001). The main probiotic microorganisms used belong to the *Bifidobacterium* and *Lactobacillus* genera (Goyal *et al.*, 2012; Reuter, 2001; Toma and Pokrotnieks, 2006). They occupy different ecological positions in the human gastrointestinal tract. Lactobacilli are normal inhabitants of the intestine, whereas, *Bifidobacteria* reside in the colon (Pedretti, 2013; Wang *et al.*, 2015). These bacteria are 'generally regarded as safe' (GRAS) because they can reside in the human body causing no harm. Some of the common probiotic microorganisms are shown in Table 2.

Table 2.2: Microorganisms used as probiotics culture

Number	***Lactobacillus* spp.**	***Bifidobacterium* spp.**	**Other Probiotic Microorganisms**
1.	*Lb. acidophilus*	*Bf. adolescenti*	*Bacillus subtilis*
2.	*Lb. casei*	*Bf. Bifdum*	*Aspergillus oryzae*
3.	*Lb. paracasei*	*Bf. Breve*	*Saccharomyces boulardii*
4.	*Lb. delbrueckii*	*Bf. Longum*	*S. cerevisiae*
5.	*Lb. helveticus*	*Bf. Infantis*	*Escherichia coli Nissle*
6.	*Lb. plantarum*	*Bf. Lactis*	*Lactococcus lactis*
7.	*Lb. gasseri*	*Bf. Animalis*	*Propionibacteria freudenreichii*
8.	*Lb. rhamnosus*	*Bf. Essensis*	*Leuconostoc mesenteroides*
9.	*Lb. johnsonii*	*Bf. Laterosporus*	*Pediococcus acidilactis*
10.	*Lb. reuteri*		*Enterococcus faecium*
11.	*Lb. fermentum*		*Streptococcus thermophiles*

Source: (Conway, 1996)

2.4.1 Lactobacilli Species

Lactobacilli have long been the most prominent probiotic microorganisms because of their association with popular fermented dairy products (Stamatova and Meurman, 2009). In healthy humans, lactobacilli are normally present in the oral cavity (10^3-10^4 CFU/g), the ileum (10^3-10^7 CFU/g), and the colon (10^4-10^8 CFU/g) (Bernardeau *et al.*, 2008). Although Lactobacilli are often described as indigenous inhabitants of the human intestinal tract, they are more likely to be autochthonous of the oral cavity or fermented foods (Taverniti *et al.*, 2014; Walter, 2008). They are also widely found in raw milk and fermented dairy products, which continue to be the preferred way to market probiotic strains in food products in the United States (Vanderhoof and Young, 2008). Beneficial effects of lactobacilli include control of intestinal inflammation, treatment of infections during pregnancy, management of allergic diseases, control of antibiotic-related diarrhea and prevention of urinary tract infections, amongst others (Bernardeau *et al.*, 2008). Several probiotic strains of microorganisms are effective in competing against common causes of travelers' diarrhea (Doron *et al.*, 2008; McFarland, 2007) caused mostly by bacterial pathogens such as *Escherichia coli*, *Campylobacter jejuni* and *Shigella* species. The most commonly used probiotic microorganisms against these pathogens are *Lb. acidophilus*, *Lb. rhamnosus GG*, *S. boulardii*, *Bf. bifidum,* and *B. coagulans*. In general, lactobacilli have received tremendous attention due to their health-promoting properties (Walter, 2008).

Lb. rhamnosus GG is the most studied probiotic *Lactobacillus* (Salminen, 1993). It was first isolated in 1987 by Gorbach and Goldin (hence GG) from the feces of a healthy

human (Gorbach *et al.*, 1987). It survives passage through the stomach and intestinal tract (Goldin *et al.*, 1992; Stansbridge *et al.*, 1993). Another useful property of this strain is the modulation of specific enzymes, for example feeding *Lb. rhamnosus* GG to healthy volunteers for four weeks decreased fecal β- glucuronidase specific activity (Goldin *et al.*, 1992). Likewise, the strains of *Lb. reuteri* are widespread in nature and can be isolated from a variety of food products, animals and the human gastrointestinal tract (Lionetti *et al.*, 2006). It appears to survive passage through the human digestive tract and persists for at least a week after stopping ingestion. *Lb. acidophilus* is also widespread in commercially available probiotic products (Fuller, 1992). It is found in fermented dairy products and is part of normal intestinal and vaginal micro-flora. Several *Lb. acidophilus* strains have been shown to produce antimicrobial substances *in vitro*, but production *in vivo* at levels high enough for a direct inhibition of pathogen growth has not been demonstrated (Sanders and Klaenhammer, 2001).

In addition, *Lb. casei* is used to make cheese and yogurt, reduce cholesterol level, enhance immune response, control diarrhea, alleviate lactose intolerance and inhibit intestinal pathogens (Mishra and Prasad, 2005). Similar to *Lb. acidophilus*, the probiotic properties of *Lb. casei* are strain-specific. *Lb. casei* strain Shirota has received much commercial attention. Its effectiveness against *Escherichia coli* was shown in mouse models for treatment of urinary tract infections (Asahara *et al.*, 2001), reduction of influenza virus in aged mice (Hori *et al.*, 2002), against *Listeria monocytogenes* infections in rats (De Waard *et al.*, 2002) and reduction of ulcer-causing *Helicobacter pylori* in humans (Sgouras *et al.*, 2004).

2.4.2 The Genus *Bifidobacteria*

Bifidobacteria are microorganisms of utmost importance in the active and complex ecosystem of the intestinal tract of humans and other warm-blooded animals, as well as of honeybees (Sgorbati *et al.*, 1995). They are distributed in various ecological niches in the human GI and genitourinary tracts, the exact ratio of which is determined mainly by the age and diet (Chen *et al.*, 2007). The indigenous microflora of infants is dominated by *Bifidobacteria*, which are established shortly after birth (Biavati *et al.*, 2000; Walker, 2013). Their proliferation is stimulated by the glycoprotein components of k-casein in human colostrum and, to a lesser extent, human milk. The number of *Bifidobacteria* decreases with the increasing age of an individual and eventually becomes the third most abundant genus after the genera *Bacteroides* and *Eubacterium* (Finegold *et al.*, 1983). The review of randomized control trials on the use of probiotics for functional constipation revealed that the favorable treatment for adults was with *Bf. lactis* (Chmielewska and Szajewska, 2010). Different studies revealed that *Bf. longum* showed prevention and treatment of necrotizing enterocolitis in newborns (Di Gioia *et al.*, 2014); reduction of radiation-induced diarrhea (Demers *et al.*, 2014); reduction of necrotizing enterocolitis with *Bifidobacteria* cocktail (*Bf. breve*, *Bf. infantis*, *Bf. bifidum*, and *Bf. longum*) (Janvier *et al.*, 2014); reduction of irritable bowel syndrome symptoms (WU *et al.*, 2013); treatment of gastrointestinal diseases (*Bf. longum*) (Yu *et al.*, 2013); perinatal intervention against onset of allergic sensitization (*Bf. longum* CCM 7952) (Schwarzer *et al.*, 2013).

Bifidobacteria are not considered true LAB, given their high G+C content and their production of a combination of lactic and acetic acid. However, they are normal inhabitants of the gastrointestinal tract of humans, making up to 25% of the cultivable fecal bacteria in adults and 80% in infants (Picard *et al.*, 2005). They are commonly used as probiotics, with a long history of safe use in fermented dairy products (Fuller, 1992). Their positive effects on human health include prevention of infection by pathogenic bacteria, immunostimulatory and anti-carcinogenic capabilities, protection against infectious diarrhea, lowering of serum cholesterol and alleviation of lactose intolerance (Russell *et al.*, 2011).

2.4.3 Other Probiotic Microorganisms

Although the term probiotic is more related to lactic acid bacteria as *Lactobacillus* and *Bifidobacterium,* it can be extended to other microorganisms that have not been explored (Parvez *et al.*, 2006; Senok *et al.*, 2005). Probiotics now include other LAB from genera such as *Streptococcus*, *Lactococcus*, *Enterococcus*, *Leuconostoc*, *Propionibacterium* and *Pediococcus* (Krasaekoopt *et al.*, 2003; O'Sullivan, 1996; Power *et al.*, 2008; Vandenplas *et al.*, 2009; Vinderola and Reinheimer, 2003). Also other non-LAB microbes are also now being used, these include bacteria such as non-pathogenic *Escherichia coli* Nissle 1917 and *Clostridium butyricum (*Harish and Varghese, 2006), yeasts (*S. cerevisiae*, *S. boulardii*), filamentous fungi (*Aspergillus oryzae*), and some spore-forming bacilli (AFRC, 1989; Mombelli and Gismondo, 2000; Wolfson, 1999).

2.5 Selection Criteria for Probiotic Strains

According to the suggestions of the WHO, FAO, and EFSA (the European Food Safety Authority), in their selection process, probiotic strains must meet safety, functionality and technological usefulness criteria (Mattila-Sandholm *et al.*, 2002; Saarela *et al.*, 2000). Therefore, the functional aspects of probiotics define their survival in the gastrointestinal tract and their immunomodulatory effect (Millette *et al.*, 2008; Tamime *et al.*, 2005). Probiotic strains have to meet the requirements associated with the technology of their production, which means that they have to be able to survive and maintain their properties throughout the storage and distribution processes (Lee, 2009). Safety aspects also include the following specifications: strains intended for human use should have a human origin and be isolated from the healthy human gastrointestinal tract and they need to have a non-pathogenic history, not associated with diseases (Lim and Im, 2009). Now, it is considered necessary to also establish the absence of transmissible antibiotic resistance genes. And also they do not deconjugate bile salts (bile salt deconjugation or dehydroxylation would be a negative trait in the small bowel (Marteau *et al.*, 1995). The theoretical basis for the selection of probiotic microorganisms including safety, functional and technological aspects is shown in Table 2.3.

Table 2.3: Selection criteria for potential probiotic lactic acid bacteria strains

Criterion	Required Properties
Safety	Human or animal origin
	Isolated from the gastrointestinal tract of healthy individuals
	History of safe use
	Precise diagnostic identification (phenotype and genotype traits)
	Absence of data regarding an association with infective disease
	Absence of the ability to cleave bile acid salts
	No adverse effects
	Lack of genes liable for antibiotic resistance localized in non-stable elements
Functionality	Competitiveness
	Ability to survive and maintain the metabolic activity, and to grow in the target site
	Resistance to low pH in the stomach
	Resistance to bile salts and enzymes
	Antagonistic activity towards pathogens
	Resistance to bacteriocins and acids produced by the endogenic intestinal microbiota
	Adherence and ability to colonize some particular sites within the host organism
Technological usability	Easy production of high biomass amounts and high productivity of cultures.
	Viability and stability of the desired properties of probiotic bacteria during the fixing process, preparation, and distribution of probiotic products
	High storage survival rate in finished products
	Guarantee of desired sensory properties of finished products
	Genetic stability
	Resistance to bacteriophages

Source: (Markowiak and Śliżewska, 2017)

The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) released a joint report in 2002 for the evaluation of probiotics. According to the report (Joint, 2002) for the strains to be classified as probiotics, the strains must be identified by phenotypic and genotypic methods, since many probiotic effects are strain-specific. The strain should be well characterized and its safety should be thoroughly evaluated *in vitro*, followed by *in vivo* studies. The efficacy of the probiotic should be evaluated by at least one human trial which should be double-blind and placebo-controlled (Dunne *et al.*, 2001). A second independent trial is preferred before the strain can be considered a probiotic and be added to food products. Thus, a potential probiotic will have to overcome harsh conditions in the GIT, such as survival at low pH, tolerance to bile acid (Hyronimus *et al.*, 2000). Once there, they must be capable of adhering to human epithelial cells. Lastly, they should prevent the colonization of human GIT by pathogenic bacteria, either by immune exclusion, competitive adhesion or synthesis of antimicrobial substances (Casula and Cutting, 2002).

2.5.1. Tolerance to the Gastric Condition

The first criteria for probiotic strain selection start with screening for acid tolerance of the potential probiotic organism (Conway *et al.*, 1987). However, the presence of food raises the pH value to around pH 3 (Erkkilä and Petäjä, 2000). The ability to survive and grow in a low pH environment is characteristic of LAB, although their tolerance mechanism is not clarified yet (Gotcheva *et al.*, 2002). Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach (Henriksson *et al.*, 1999). Numerous *in vitro* and *in vivo* studies have demonstrated that probiotics organisms can

survive in the gastric transit where the cells as exposed to pH values <2.0, though the time exposure (1 to 2 h) is relatively short (Dunne *et al.*, 2001; Singh *et al.*, 2014). Survival of lactobacilli in the acidic environment has also been enhanced in the presence of the metabolized sugar that allows the cell membrane proton pumps to operate and prevent the lowering of intracellular pH (Binns, 2013).

Likewise, Syal and Vohra (2013) isolated twenty yeast strains from traditional Indian fermented foods (*idli* and *jalebi* batter) and screened them for various probiotic properties. The results showed that seven of these isolates survived in conditions similar to the gut with a survival rate as high as 100% at pH 2.0-2.5. In line with this, Compaoré *et al.* (2013) isolated thirteen species of *Bacillus* spp. including six *B. subtilis* sp. *subtilis*, four *B. licheniformis* and three *B. amyloliquefaciens* isolated from traditional *bikalga* and the results indicated that all the thirteen isolates showed a good rate of survival at pH 2.5.

2.5.2 Tolerance to the Bile Acid

Bile secreted in the small intestine reduces the survival of bacteria by destroying their cell membranes, the major components of which are lipids and fatty acids; these modifications may affect not only the cell permeability and viability but also the interactions between the membrane and the environment (Succi *et al.*, 2005). Resistance to bile salts is considered as an important parameter for selecting probiotic strains. A concentration of 0.15-0.3% of bile salt has been recommended as a suitable concentration for selecting probiotic bacteria for human use (Gibson and Roberfroid, 1995). Bile is synthesized in the liver from cholesterol and is secreted from the gall bladder into the duodenum in the conjugated form in volumes ranging from 500 to700 ml per day

(Mourad and Nour-Eddine, 2006). The relevant physiological concentrations of human bile range from 0.1 to 0.3% (Dunne *et al.*, 2001) and 0.5% (Mathara *et al.*, 2008). All isolate demonstrated good capacity to resist bile salts by presenting the surviving percentage greater than 50% under exposure to 0.2% bile salts after 24h at 37°C (Sieladie *et al.*, 2011). In line with this, Hoque *et al.* (2010) isolated *Lactobacillus* spp. from yogurt (Bogra and Khulna regions of Bangladesh) are able to tolerate up to 0.3% of bile concentrations.

2.5.3 Antimicrobial Activity

Antimicrobial activity is one of the most important selection criteria for probiotics (Leite *et al.*, 2015). The probiotic strain producing antimicrobial substances is most important in developing the probiotic supplement and probiotic-rich foods. Antagonistic effects of LAB are materialized by producing some substances such as organic acids, carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins (Chelule *et al.*, 2010; Ouwehand and Vesterlund, 2004).

Furthermore, acids from bacteria are generally thought to exert their antimicrobial effect by interfering with the maintenance of cell membrane potential, inhibiting active transport, reducing intracellular pH and inhibiting a variety of metabolic functions (Doores, 1993). They have a very broad mode of action and inhibit both Gram-positive and Gram-negative bacteria as well as yeast and molds (Jampaphaeng *et al.*, 2019; Pehrson *et al.*, 2015). One good example is propionic acid produced by propionic acid bacteria, which has formed the basis for some bio preservative products, given its antimicrobial action against microorganisms including yeast and molds. In addition to

acids, LAB strains can produce a range of other antimicrobial metabolites such as ethanol from the heterofermentative pathway, H_2O_2 produced during aerobic growth and diacetyl which is generated from excess pyruvate coming from citrate (Yang and Moon, 2018). In particular, H_2O_2 can have a strong oxidizing effect on membrane lipids and cellular proteins and is produced using such enzymes as the flavoprotein oxidoreductases NADH peroxidase, NADH oxidase and α-glycerophosphate oxidase (König and Fröhlich, 2017).

Other examples of antimicrobial substances produced by LAB which have antagonistic activity include bacteriocins. Many members of LAB produce a range of antimicrobial peptides and proteins which are collectively referred to as bacteriocins (Gänzle and Salovaara, 2019). Bacteriocins with specific inhibitory activity against closely related species are most studied (Musikasang *et al.*, 2012). At present, nisin (produced by the *Lb. lactis subsp. lactis strains)* is a well-known and purified bacteriocin approved for use in the product intended for human consumption (Cleveland *et al.*, 2001). Nisin is active mainly against *Staphylococcus, Micrococcus* and *Listeria* species. Other probiotic strains also produce various kind of bacteriocins which have an adversative effect on a various pathogenic organism. Lactococcin, Salivarcin, Acidocin 8912, Plantarcin and Lacticin A are produced by *Lc. lactis, Streptococcus salavarius, Lb. acidophilus, Lb. plantarum* and *Lb. delbrueckii,* respectively (Dash, 2009; Mishra and Prasad, 2000). These bacteriocins can potentially be used to control the growth of spoilage and pathogenic organisms in food (Rodríguez *et al.*, 2000). Bacteriocin producing lactococcal strains have been used successfully as starter cultures for cheese making in order to improve the safety and quality of the cheese. In previous work, 79 wild lactococci have been studied and 32 of these have been found to be antimicrobially active (Wouters *et al.*, 2002). In 17 of these

strains, the well-known antimicrobial peptide nisin was found, whereas the others produced diplococcin, lactococcin or an unidentified bacteriocin-like compound. Moreover, the use of nisin as an effective preservative in processed cheese has been widely accepted (Delves- Broughton, 1990).

2.5.4 Antibiotic Resistances

Antibiotic resistance has become a global concern as a result of the increasing use of antibiotics in the treatment of microbial infection of humans as well as animals (Austin *et al.*, 1999; Robredo *et al.*, 2000). Nowadays, antibiotic resistance is considered a serious concern in medicine. Antibiotic resistance in bacteria may be intrinsic or acquired (Authority, 2012). Intrinsic resistance is a naturally occurring trait and may be considered as a species characteristic, whereas acquired resistance derives either from genetic mutations or acquisition of foreign DNA from other bacteria (Abriouel *et al.*, 2017; Authority, 2012).

Lactobacilli display a wide range of antibiotic resistances naturally (Charteris *et al.*, 1998b), but in most cases, antibiotic resistance is not of the transmissible type. *Lactobacillus* strains with non-transmissible antibiotic resistances do not usually form a safety concern. Several species of lactobacilli including *Lb. rhamnosus* and *Lb. casei* are intrinsically resistant to vancomycin (Charteris *et al.*, 1998b; Nicas *et al.*, 1989; Swenson *et al.*, 1990). These species have peptidoglycan precursors terminating with D-lactate instead of the target precursor for vancomycin activity terminating with D-alanine (Billot-Klein *et al.*, 1994). Many intrinsically vancomycin-resistant strains of lactobacilli have a long history of safe use as probiotics and there is no indication that vancomycin-

resistant lactobacilli could transfer the resistance to other bacteria. The vancomycin resistance factor of the probiotic strain *Lb. rhamnosus* GG is not closely related to those of enterococci, and they could not observe the transfer of antibiotic resistance between *Lb. rhamnosus* GG and enterococci (Tynkkynen *et al.*, 1998).

Likewise, most *Bifidobacteria* are intrinsically resistant to nalidixic acid, neomycin, kanamycin, gentamycin, streptomycin and metronidazole (Charteris *et al.*, 1998a; Miller and Finegold, 1967). In earlier studies vancomycin has been found highly inhibitory against *Bifidobacteria* (Lim *et al.*, 1993; Miller and Finegold, 1967), whereas in a recent study by Charteris *et al.* (1998a) vancomycin resistance was suggested to be a general characteristic of *Bifidobacteria*. However, differences in the techniques used in susceptibility testing hinder the comparison of data. Suppression of fecal counts of *Bifidobacteria* during vancomycin therapy would suggest susceptibility of intestinal *Bifidobacteria* to this agent (Edlund *et al.*, 1997).

2.5.5 Proteolytic Activity

The ability to produce extracellular proteinases is a very important feature of LAB. These proteinases catalyze the initial steps in the hydrolysis of milk proteins, providing the cell with the amino acids that are essential for the growth of probiotics. The proteolytic activities of probiotic organisms were studied extensively and proteolytic enzymes were isolated and characterized. Bacterial enzymatic hydrolysis was shown to enhance the bio-availability of protein and fat (Friend and Shahani, 1984). Bacterial protease can increase the production of free amino acids which can benefit the nutritional status of the host particularly if the host has a deficiency in endogenous protease production. This ability of

probiotics has become even more important upon realizing that a range of bioactive peptides may be liberated due to microbial action (Friend and Shahani, 1984).

2.5.6 Exopolysaccharide Production

Many microorganisms have been reported to produce exopolysaccharides (EPS), either in the form of capsules, or slime secreted into the extracellular environment (Sanni *et al.*, 2002). Microbial polysaccharides could provide a potential source of biopolymers for food and other industries (Gassem *et al.*, 1997). Therefore, apart from the production of lactic acid, flavoring compounds and bacteriocin like substances, several strains of LAB secrete extracellular polysaccharide in a favorable environment (PV *et al.*, 2009). The term EPS is used to describe extracellular polysaccharide either attached as a capsule with bacterial cell wall or liberated into the medium as ropy polysaccharide (Sutherland, 1972). The EPS plays an important role in the improvement of the physical properties of fermented milk, which act like a food stabilizer, viscosifier, emulsifier or gelling agent providing a product with natural thickness (Ruas-Madiedo, 2005).

Generally, numerous LAB, with some strains of *Bifidobacteria* are reported to produce EPS (Salazar *et al.*, 2009). Most of them belong to the genera of *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus*. The EPS producing LAB have been traditionally used in the Scandinavian fermented milk products to impart desirable texture and rheological properties (Macura and Townsley, 1984). The products made with ropy strains have a smooth body, high viscosity, and less syneresis than the products made with non-ropy strains (Wacher-Rodarte *et al.*, 1993). Often in the literature, to describe different EPS producing phenotypes, the term ropy, mucoid and

slime have been interchangeably used. However, not all mucoid or slime producing cultures are ropy. The ropy colonies are able to form strand when touched with an inoculating loop, whereas, mucoid colonies have glistening and slimy appearance on agar plates and are not able to produce strands by this method (Vescovo *et al.*, 1989).

2.5.7 Adhesion Properties of Probiotics

Adhesion of probiotic strains to the intestinal surface and the subsequent colonization of the human GI-tract have been suggested as an important prerequisite for probiotic action. Adherent strains of probiotic bacteria are likely to persist longer in the intestinal tract and thus have better possibilities of showing metabolic and immunomodulatory effects than non-adhering strains. Adhesion provides an interaction with the mucosal surface facilitating contact with gut-associated lymphoid tissue mediating local and systemic immune effects. Thus, only adherent probiotics have been thought to effectively induce immune effects and to stabilize the intestinal mucosal barrier (Tuomola and Salminen, 1998). Adhesion may also provide means of competitive exclusion of pathogenic bacteria from the intestinal epithelium: Exclusion of pathogens by LAB has been shown *in vitro* using Caco-2 and HT-29-MTX cell lines (Bernet *et al.*, 1993). In the inhibition of pathogen adhesion *in vitro* both living and heat-killed *Lb. acidophilus* cells have been effective (Coconnier *et al.*, 1993). Similarly, El-Jeni *et al.* (2015) reported that *Leu. mesenteroides* and *Ent. faecium* strains were able to adhere, *in vitro*, to the stainless steel plates, with variable rates. Some studies also confirmed that adhesion of *Ent. faecium* strains to stainless steel was considered as selection criteria of potential probiotic characteristics (Giaouris *et al.*, 2005; Paramithiotis *et al.*, 2006).

Although a lot of research effort has been carried out on probiotic adhesion studies, the role of adhesion in successful probiotic function remains speculative (Saarela *et al.*, 2000). It could also be argued that strong adhesion ability may increase the risk of infection in the host. Some probiotic strains are poorly adhering *in vitro* and: or *in vivo* and still, they can show positive effects in the hosts. The reproducibility of *in vitro* adhesion studies can be poor (especially between different laboratories), which also complicates the interpretation of the results.

2.6 Mechanisms of Probiotic Action

The mechanisms by which probiotics exert biological effects are still poorly understood, but the nonspecific terms such as colonization resistance or competitive exclusion are often used to explain their mode of action (Elo *et al.*, 1991). Colonization resistance or competitive exclusion describes a phenomenon whereby the indigenous anaerobic flora limits the concentration of potentially pathogenic (mostly aerobic) flora in the digestive tract (Vollaard and Clasener, 1994). The concept of competitive exclusion was first developed during the early 1970s when it was discovered that the administration of mixed adult intestinal microorganisms conferred adult-type resistance against *Salmonella* infection to newly hatched chicks (Nurmi *et al.*, 1992).

In general, the effects of probiotics may be classified into three modes of action (Oelschlaeger, 2010). These are: (1) Probiotics might be able to modulate the host's defenses including the innate as well as the acquired immune system. This mode of action is most likely important for the prevention and therapy of infectious diseases but also for the treatment of (chronic) inflammation of the digestive tract or parts thereof. In

addition, this probiotic action could be important for the eradication of neoplastic host cells; (2) Probiotics can also have a direct effect on other microorganisms, commensal and/or pathogenic ones. This principle is in many cases is important for the prevention and therapy of infections and restoration of the microbial equilibrium in the gut; (3) Probiotic effects may be based on actions affecting microbial products like toxins and host products, *e.g.* bile salts and food ingredients. Such actions may result in the inactivation of toxins and detoxification of host and food components in the gut.

2.7 Health Benefits of Probiotics

Probiotic bacteria are helpful in maintaining good health and in fighting intestinal and other disease orders when consumed as a food component (Hussain, 2013). There is significant number of studies indicating the benefits of probiotics that include reducing antibiotic-associated diarrhea, allergy, lactose intolerance, prevention of colon cancer, prevention of eczema in children, reduction of cholesterol as well as development of immune system and protection against gut pathogens (Jankovic *et al.*, 2010; Rijkers *et al.*, 2010).

2.7.1 Stimulation of the host immune system

A number of studies have been performed *in vitro* and in animals (Gill *et al.*, 2000) which clearly show that probiotic strains can modify immune parameters. Correlating these findings with events taking place in the human body is still somewhat unclear, but evidence is mounting that such effects occur. In a series of randomized, double-blind, placebo-controlled clinical trials, it was demonstrated that dietary consumption of *B.*

lactis HN019 and *Lb. rhamnosus* HN001 resulted in measurable enhancement of immune parameters in the elderly. Probiotic modulation of host immunity is a very promising area for research. Supportive data is emerging, such as those carried out in humans showing that probiotic microorganisms can enhance natural killer (NK) cell activity in the elderly and nonspecific host defenses can be modulated (Gill *et al.*, 2001).

There is a need to specify whether the activities being advocated are designed to operate in otherwise healthy people or subjects with known diseases. Some of the critical factors involved in the host's defenses have been identified and include the induction of mucus production or macrophage activation by lactobacilli signaling (Merk *et al.*, 2005; Miettinen *et al.*, 2000). It is also recognized that in some situations, stimulation of factors such as inflammatory cytokines may confer health benefits on the host (Gardiner *et al.*, 2002).

2.7.2 Inhibition of Pathogenic Bacteria

There has been considerable research on the anti-pathogenic activity of probiotics or a probiotic mixture (Kerry *et al.*, 2018). Tejero-Sariñena *et al.* (2013) investigated the influence of probiotics on the survival of *S.* Typhimurium and *C. difficile* in an *in vitro* model and postulated that probiotics inhibit pathogens by the production of short-chain fatty acids (SCFAs). SCFAs help to maintain an appropriate pH in the colonic lumen, which is imperative in the expression of numerous bacterial enzymes and in metabolism of foreign compounds and carcinogens in the gut (Kareem *et al.*, 2014).

Islam (2016) also suggested that a wide variety of anti-pathogenic compounds, like bacteriocins, ethanol, organic acids, diacetyl, acetaldehydes, and peptides are produced by many probiotics. Among these compounds, peptides and bacteriocins, in particular, are mostly involved in increasing the membrane permeability of the target cells, which leads to the depolarization of the membrane potential and, ultimately, cell death (Simova *et al.*, 2009). Similarly, the production of H_2O_2 by these bacterial groups causes the oxidation of sulfhydryl groups, resulting in the denaturation of several enzymes results in the peroxidation of membrane lipids, thus, increasing membrane permeability of the pathogenic microorganisms and consequently, cell death (Ammor *et al.*, 2006). Some of these compounds may act by lowering pH by organic acids like lactic and acetic acids (Kareem *et al.*, 2014).

In addition to producing anti-pathogenic bioactive compounds that directly affect pathogens, probiotics also stimulate host anti-pathogenic defense pathways, such as stimulating or activating the pathway involved in the production of defenses that are cationic anti-microbial peptides (Figueroa-González *et al.*, 2011). Another mechanism by which probiotics exert their anti-pathogenic activity is by competing for pathogen binding and receptor sites, as well as for available nutrients and growth (Bermudez-Brito *et al.*, 2012).

2.7.3 Alleviation of Symptoms of Lactose Intolerance

A large number of population become lactose intolerant after weaning (Kim and Gilliland, 1983; Matthews *et al.*, 2005). These lactose-intolerant people cannot metabolize lactose due to the lack of essential enzyme β-galactosidase (Kim and

Gilliland, 1983). When they consume milk or lactose-containing products, symptoms including abdominal pain, bloating, flatulence and diarrhea ensue (Levri *et al.*, 2005). The studies provide that the addition of certain starter cultures to milk products allows the lactose-intolerant people to consume those products without the usual rise of breath hydrogen or associated symptoms (Lin *et al.*, 1991). The beneficial effects of probiotics on lactose intolerance are due to the high lactase activity of bacterial preparations used in the production and increased lactase enzyme (Salminen *et al.*, 2004). Furthermore, the *Lb. bulgaricus* and *Strep. thermophilus* which are used to produce yogurt, are not resistant to gastric acidity. Hence, the products with probiotic bacteria are more efficient for lactose-intolerant people because of their ability to withstand low pH and capability to improve the digestibility of lactose by β-galactosidase production (Kim and Gilliland, 1983).

2.7.4 Reduction of Serum Cholesterol

There are claims that consumption of fermented milk significantly reduces serum cholesterol (Gilliland, 1989; Yadav *et al.*, 2019). For hypercholesterolemic individuals, significant reductions in plasma cholesterol levels are associated with a significant reduction in the risk of heart attacks (Yadav *et al.*, 2019). The principal site of cholesterol metabolism is the liver, although appreciable amounts are formed in the intestines. Claims are strong that certain *Lb. acidophilus* strains and some *Bifidobacteria* species are able to lower cholesterol levels within the intestine (Miremadi *et al.*, 2014). Cholesterol co-precipitates with deconjugated bile salts as the pH declines as a consequence of lactic acid production by LAB (Marshall, 1996). The role that *Bifidobacteria* cultures may play

in lowering serum cholesterol is not yet understood. Park *et al.* (2018) reports on various experiments that conclude that a factor is produced in the fermented milk that inhibits cholesterol synthesis in the body.

Another theory is that *Lb. acidophilus* deconjugates bile acids into free acids, which are excreted more rapidly from the intestinal tract than are conjugated bile acids (Lourens-Hattingh and Viljoen, 2001). As free bile salts are excreted from the body, the synthesis of new bile acids from cholesterol can reduce the total cholesterol concentration in the body (Gilliland and Speck, 1977b). A third hypothesis is that reduction of cholesterol may also be due to co-precipitation of cholesterol with deconjugated bile salts at lower pH values as a result of lactic acid production by the bacteria (El-Gawad *et al.*, 2005; Kailasapathy and Rybka, 1997).

2.7.5 Reduction of Antibiotic-associated Diarrhea

Antibiotic-associated diarrhea (AAD) is a frequent complication observed both in the outpatient and inpatient settings that develops in up to a third of all patients treated with antibiotics (Barbut and Meynard, 2002), and in particular in 11–62% of children and in up to 80% of hospitalized toddlers (Turck *et al.*, 2003). Antibiotic-associated diarrhea is normally defined as 3 or more liquid stools in 24 h that occur in subjects during or even within 6–8 weeks after antibiotic treatment (McFarland, 1998). The use of antibiotics disturbs the gastrointestinal flora and causes a range of clinical symptoms, most notably diarrhea. The symptoms range from mild and self-limiting to severe, and AAD is an important reason for non-adherence with antibiotic treatment (Xu *et al.*, 2017).

Mild or severe episodes of diarrhea are common side effects of antibiotic therapy as the normal microflora tends to be suppressed. The spectrum may range from diarrhea without mucosal abnormality to pseudomembranous colitis. The latter is a severe form of antibiotic-associated diarrhea (caused by *C. difficile*, cytotoxic strains of which may emerge after antibiotic use) (Goldin, 1998). The name of the condition is derived from the plaque-like adhesion of fibrinopurulent material to the damaged mucosal layer and it is characterized by diarrhea, abdominal distention, vomiting, fever, and leukocytosis and if untreated might lead to complications such as toxic megacolon and perforation (Hempel *et al.*, 2012).

Treatment with probiotics might maintain or restore gastrointestinal micro-ecology during or after antibiotic treatment through competition of receptor or nutrients, inhibition of epithelial and mucosal adherence, introduction of lower pH, stimulation of immunity, or production of antimicrobial substances (Cremonini *et al.*, 2002; Rolfe, 2000). One previous meta-analysis assessed the efficacy of probiotics for the prevention of pediatric AAD, with the combined results in favor of probiotic co-administration with antibiotics (Johnston *et al.*, 2006). Several studies that have been carried out suggest that probiotic use is associated with a reduced risk of antibiotic-associated diarrhea (McFarland, 2006; Sazawal *et al.*, 2006). Meta-analysis evaluating the available evidence on probiotics for the prevention and treatment of antibiotic-associated diarrhea concluded that probiotic administration (*Lb. rhamnosus, Lb. casei* and the yeast *S. boulardii*, as these are the probiotics predominantly included in the majority of trials) is associated with a reduced risk of the condition (Beausoleil *et al.*, 2007). Furthermore, many clinical trials have also assessed the efficacy of probiotics for the prevention and treatment of

pediatric AAD in China. The majority of probiotics used in these trials were *Bifidobacterium*-based interventions in combination with other genera (Xu *et al.*, 2017).

2.7.6 Prevention of Colon Cancer

Colon cancer is one of the most common and most diagnosed types of cancer. Colon cancer prevalence has rapidly increased as dietary patterns have modified to blending low carbohydrate, high protein, low fiber, and high fat (Lee *et al.*, 2008). An ever-increasing literature demonstrates that the microbiota can have a strong influence on colon cancer prevention and on the healing process during intestinal inflammation. The intestinal microbiota can influence many aspects of intestinal health, including its cellular features, physiology, metabolism, development, and immune homeostasis. Therefore, modifying the intestinal microbiota composition by probiotics when ingested in adequate amounts may prevent the development of colorectal cancer because these microorganisms both have an influence on the microbiota and potentially afford health benefits to the host (dos Reis *et al.*, 2017).

Natural sources that confer anti-carcinogenic effects for the prevention of colon cancer, such as probiotics, have been receiving important focus in recent years (Gayathri and Rashmi, 2016). Among many available probiotic strains widely used clinically, specific Lactobacilli and *Bifidobacteria* are well characterized in terms of their activity and anti-inflammatory role in modulating cytokine production in human dendritic cells (Dong *et al.*, 2012; Evrard *et al.*, 2011). . Bacterial enzymes (*β*-glucuronidase, nitroreductase, and azoreductase) convert procarcinogens to active carcinogens in the colon (Aso and Akazan, 1992). However, according to some studies probiotics reduce the risk of cancer

by decreasing bacterial enzyme activity (Aso *et al.*, 1995; Aso and Akazan, 1992). Although the exact mechanism for the anti-tumor action is not known, some suggestions were proposed by many authors as follows: (1) Carcinogen/procarcinogens are suppressed by binding, blocking or removal; (2) Suppressing the growth of bacteria with enzyme activities that may convert the procarcinogens to carcinogens; (3) Changing the intestinal pH thus altering microflora activity and bile solubility; (4) Altering colonic transit time to remove fecal mutagens more efficiently; (5) Stimulating the immune system (Fooks and Gibson, 2002; Scheinbach, 1998).

There are *in vitro* and *in vivo* evidence not only from animal studies but also from human studies that probiotics have beneficial effects on suppression of cancer (Aso and Akazan, 1992). The consumption of a large quantity of dairy products containing *Lactobacillus* or *Bifidobacterium* may be related to a lower occurrence of colon cancer (Hirayama and Rafter, 2000; Rafter, 2003). Likewise, the regular consumption of *Lb. plantarum*, *Lb. acidophilus* and *B. longum* at a high dosage for 16 days increased the diversity and microbial richness in individuals with colorectal cancer (CRC) undergoing a colorectomy (Liu *et al.*, 2011). A number of studies have shown that predisposing factors like "increase in enzyme activity that activate carcinogens, increase procarcinogenic chemicals within the colon or alter population of certain bacterial genera and species" are altered positively by consumption of certain probiotics (Brady *et al.*, 2000).

2.7.7 Prevention of Eczema in Children

The prevalence of allergic diseases has increased over the last 35–40 years, particularly in Western societies. Although research is preliminary on how probiotics might modulate

allergic reaction, they may exert a beneficial effect by improving mucosal barrier function and microbial stimulation immune system (Macfarlane and Cummings, 2002). Probiotic bacteria are important in downregulating inflammation associated with hypersensitivity reactions in patients with atopic eczema (Parvez *et al.*, 2006). Perinatal administration of *Lb. rhamnosus GG* decreased subsequent occurrence of eczema in at-risk infants by one-half (Isolauri *et al.*, 2000). In newborn infants, the initial bacteria to colonize the sterile GI tract may establish a permanent niche and have a lasting impact on immune regulation and subsequent development of atopic disorders. It was suggested that probiotics may enhance endogenous barrier mechanisms of the gut and alleviate intestinal inflammation, providing a useful tool for treating food allergy (Macfarlane and Cummings, 2002).

Probiotics may also be helpful in alleviating some of the symptoms of food allergies such as those associated with milk protein. Possibly by degrading these proteins to smaller peptides and amino acids, added to the diet of infants on a hydrolyzed whey formula decreased the symptoms of atopic dermatitis (Majamaa and Isolauri, 1997). Probiotics have also been found to upregulate anti-inflammatory cytokines, such as interleukin, in atopic children (Majamaa and Isolauri, 1997). This is seen both as an immunostimulatory effect in healthy subjects and as a down-regulation effect of immunoinflammatory responses in hypersensitive patients (Ricci *et al.*, 2016). Similarly, in animal models, it has been demonstrated that probiotics reinforce mucosal degradation of antigens by enhancing the breakdown of macromolecules (Pessi *et al.*, 1998).

2.7.8 Protection Against Autism Depression

In recent years, many studies have been devoted towards elucidating the influence of gut microbiota on the CNS (Dinan and Cryan, 2017). The "microbiota-gut-brain axis" is an interactive, bi-directional communication established by the exchange of regulatory signals between the GIT and CNS (Dinan and Cryan, 2017; Mayer *et al.*, 2015). The effect of probiotics on the central nervous system (CNS) has been mainly studied in clinical trials, where it has been evident that gut microbiota influence human brain development function (Tillisch, 2014). In children with autism spectrum disorder, a daily dose of *Lb. plantarum* WCFS1 ($4.5x10^{10}$ CFU/ day) led to an improvement in their school records and attitude towards food (Umbrello and Esposito, 2016).

Messaoudi *et al.* (2011) discussed reduced psychological distress in a randomized trial involving healthy volunteers treated with oral administration of *Lb. helveticus* R0052 and *Bf. longum* R0175. Another clinical trial showed a decrease in anxiety symptoms by administration of *Lb. casei* strain *Shirota* to patients suffering from chronic fatigue syndrome (Rao *et al.*, 2009). However, despite an increase in the *Lactobacillus* and *Bifidobacteria* levels, the bowel functions were not studied. Hence, it is feasible that the reduced anxiety was due to improved bowel function. Szajewska (2015) reported that autism spectrum and attention-deficit/hyperactivity disorders in children could be prevented by *Lb. rhamnosus* administration to the mother at 4 weeks from expected delivery. Human intestinally derived strains of *Lb. brevis* DPC6108 and *Bf. dentium* were reported to produce large amounts of g-aminobutyric acid, a brain neurotransmitter that helps humans to suppress anxiety and depression (Dinan and Cryan, 2017).

2.7.9 Protection Against Heavy Metal Toxicity

Heavy metal toxicity is one of the oldest environmental problems and remains a serious health concern today. Cadmium (Cd) and lead (Pb) are common toxic heavy metals in the environment (Zhai *et al.*, 2015). The general public is exposed to Cd and Pb through ambient air, drinking water, food, industrial materials, and consumer products (Klaassen and Watkins, 1996; Nordberg *et al.*). However, probiotics have been reported to play important roles in the alleviation or prevention of Cd and Pb toxicity (Monachese *et al.*, 2012). Some species of lactic acid bacteria including *Lb. rhamnosus*, *Lb. plantarum* and *Bf. longum* are capable of binding heavy metals *in vitro* (Halttunen *et al.*, 2008; Halttunen *et al.*, 2007). Lactic acid bacteria are known to have anti-oxidative properties in human subjects (Ejtahed *et al.*, 2012; Kullisaar *et al.*, 2003), which may be another important characteristic of heavy metal toxicity protection.

Dietary supplements have been reported to play important roles in the alleviation or prevention of Cd and Pb toxicity (Zhai *et al.*, 2015). *Lb. plantarum* CCFM8610 with a good Cd binding capacity is able to protect mice from acute and chronic Cd toxicity via its intestinal sequestration and antioxidant effects (Zhai *et al.*, 2014; Zhai *et al.*, 2013). Hence, the oral administration of this strain effectively decreased intestinal Cd absorption, reduced Cd accumulation in tissue, alleviated tissue oxidative stress, reversed hepatic and renal damage, and ameliorated the corresponding histopathological changes of Cd-exposed mice. *Lb. plantarum* CCFM8661 protects against Pb toxicity by recovering the blood activity, decreasing the Pb levels in the blood and tissues and preventing Pb-induced oxidative stress (Tian *et al.*, 2012).

Several recent reports confirmed that other probiotics strains may also be protective against heavy metal toxicity (Bisanz *et al.*, 2014; Zhai *et al.*, 2016). A mixture of *Lb. rhamnosus* Rosell-11, *Lb. acidophilus* Rosell-52 and *Bf. longum* Rosell-175 significantly reduced Cd-induced genotoxicity both *in vitro* using liver tissue culture and in rats (Jama *et al.*, 2012). Another study investigated the potential of *Lb. rhamnosus* GR-1 supplemented yogurt to lower heavy metal levels in at-risk populations of pregnant women and in children in Tanzania (Bisanz *et al.*, 2014). The results of the same workers showed that blood levels of mercury and arsenic of pregnant women increased in the control groups ($p<0.05$) but remained stable in the probiotic group, and this indicates a protective effect of *Lb. rhamnosus* GR-1 consumption. This means that with confirmed protection against heavy metal toxicity in animal studies, probiotics also have the potential to treat heavy metal toxicity in humans. These studies indicate that specific probiotic or cocktails of probiotic mixes may be required for protection against different types of heavy metal toxicity. Therefore, the use of these probiotic lactobacilli can be considered a new dietary therapeutic strategy against heavy metal toxicity (Zhai *et al.*, 2015; Zhai *et al.*, 2016).

Chapter 3 *In Vitro* Evaluation of Probiotic Properties of Lactic Acid Bacteria Isolated from some Traditionally Fermented Ethiopian Food Products

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Abstract

Probiotics are live microorganisms which when administered in adequate amounts promote the health of the consumer. The aim of this study was to evaluate *in vitro* probiotic properties of LAB isolated from traditional Ethiopian fermented *teff* dough, *ergo* and *kocho* products. A total of 450 LAB were isolated, of which 34 (7.56%) isolates showed 45.35- 96.33% and 33.33- 91.75% survival rate at pH 2 for 3 and 6 h in that order. The 34 acid-tolerant isolates also survived to 0.3% bile salt tolerance test for 24 h with 82.58 to 99.44% rate of survival. The acid and bile salt-tolerant LAB isolates could also inhibit some reference foodborne pathogenic bacteria to varying degrees. Hence, among the 34 acid-and-bile salt-tolerant isolates, 9 (26.47%) were able to produce bacteriocins to varying degrees. All the 34 acid and bile salt-tolerant isolates were susceptible to ampicillin, tetracycline and erythromycin, however, they showed resistant to kanamycin. Seventeen of the 34 isolates were found sensitive/resistant to streptomycin. The *in vitro* adherence to stainless steel plates of the 34 potential probiotic LAB isolates

ranged from 29.21 to 41.94% adhesion rate. The identification of four selected probiotic LAB strains and their genetic relatedness was performed based on 16S rRNA gene sequence comparisons. These isolates belonged to *Lactobacillus* species including *Lb. plantarum*, *Lb. paracasei* subsp. *tolerans*, *Lb. paracasei* and *Lb. plantarum*. The remaining 30 potential probiotic LAB strains and their genetic relatedness was performed based on whole-genome sequence comparisons. Accordingly, these isolates belonged to *Lactobacillus* and *Lactococcus* species including 22 *Lb. plantarum*, 1 *Lb. paracasei*, 1 *Lb. brevis* and 6 *Lc. lactis*. Among the 30 potential probiotic LAB strains, BAGEL predicted 1 bacteriocin for class III; 7 bacteriocins for class II and 3 bacteriocins for class I in the genome of 9 strains. The 34 *Lactobacillus* and *Lactococcus* strains were found to be potentially useful to produce functional products that contain probiotics.

Keywords/ Phrases: *Ergo*, *Teff* dough, *Kocho*, *Lactobacillus*, *Lactococcus*

3.1 Introduction

A variety of fermented food products are produced worldwide, which contribute significantly to the diets of many people (Nath *et al.*, 2016). Fermented food products are used to describe a special class of the food products characterized by various kinds of biomolecules breakdowns in the presence of probiotic microorganisms, but seldom is carbohydrate the only constituent acted upon (Hasan *et al.*, 2014). Fermented food and beverage products have emerged as not only the source of nutrition but also as functional and probiotic foods, which besides nutritional value have health effects or provide protection against foodborne diseases (Bourdichon *et al.*, 2012).

The problem of foodborne diseases (FBD) is multifactorial and their prevention and control require multidisciplinary approaches that involve human beneficial live microbes (probiotics) in order to combat these pathogens and their associated health risks (Sivapalasingam *et al.*, 2004). Several *in vitro* studies indicate that the growth of foodborne pathogenic microbes is inhibited by probiotic lactic acid bacteria (Anteneh Tesfaye *et al.*, 2011; Dowarah *et al.*, 2018; Girum Tadesse *et al.*, 2005). The consumption of a large number of probiotics lives microorganisms together with food fundamentally promote the health of the consumers (Hill *et al.*, 2014). LAB are a diverse group of microorganisms consisting of Gram-positive, aerotolerant, acid-tolerant, usually non-sporulation and non-respiring rod or cocci microorganisms and play an important role in the process of fermentation of food by inhibiting spoilage/pathogenic bacteria and by producing excellent flavor, aroma and texture of fermented foods (Akkoc *et al.*, 2011; O'Bryan *et al.*, 2015). Some LAB are probiotics, others may be potential probiotics or just fermentation cultures that are widely distributed in nature and can be used in the food industry (Ricci *et al.*, 2019).

Lactic acid bacteria could be isolated from many kinds of sources such as milk products, fermented foods, animal intestines or freshwater fishes, soil samples, sugar cane plants, and poultry farms (Barakat *et al.*, 2011). The most common types of probiotic lactic acid bacteria include different *Lactobacillus* spp. (*Lb. acidophilus, Lb. johnsonii, Lb. casei, Lb. rhamnosus, Lb. gasseri* and *Lb. reuteri*) and genus *Bifidobacteria* (*Bf. bifidum, Bf. animalis subsp. lactis, Bf. longum* subsp. *longum* and *Bf. longum* subsp. *infantis*) (Arthure *et al.*, 2002; Chassard *et al.*, 2011). Lactic acid bacteria are also useful in the treatment of various diseases caused by drug-resistant pathogenic microbes (Marco *et al.*, 2017).

Probiotic microbes may provide nutrients, enhance growth, produce enzymes, inhibit pathogens and enhance immune responses (Manhar *et al.*, 2016).

In Ethiopia, traditionally fermented food products are prepared at the household level and the consumption of these fermented food products is commonly practiced. Thus, some studies on isolation and screening of antibacterial producing LAB from traditionally fermented foods were undertaken by many workers (Fitsum Tigu *et al.*, 2016; Negasi Akalu *et al.*, 2017). Likewise, Anteneh Tesfaye *et al.* (2011) have revealed that the antagonistic effect of lactic acid bacterial strains either as pure or mixed-cultures against some foodborne pathogens during fermentation and storage of fermented milk. However, there are still few research data available on the characterization of probiotic LAB. Most of the traditionally fermented products of Ethiopia are consumed without further heat processing which can be considered as ideal vehicles to carry probiotic bacteria into the human gastrointestinal tract. Probiotic strains isolated from traditionally fermented foods and drinks could have application as a starter culture for large scale production of the traditional product and have a desirable functional property for their application as probiotics against foodborne pathogens. Therefore, this study attempts to evaluate the *in vitro* probiotic properties of LAB isolated from three traditional fermented food products such as *teff* dough, *ergo,* and *kocho* with respect to their potential probiotic properties against some foodborne pathogens.

3.2 Materials and Methods

3.2.1 Sample Collection

A total of 45 samples that consisted of fifteen samples for each of traditional fermented food products (*teff* dough, *kocho* and *ergo*) were obtained from Addis Ababa City and its surroundings (Figure 3.2). Each sample (200 g/ml) was aseptically collected by using sterilized containers. The samples were brought to the laboratory in an icebox and stored in a refrigerator at +4°C until further analysis was carried out. *Ergo* is a locally fermented milk product. *Teff* dough is made by fermenting *teff* (*Eragrostis tef*) flour which is used to prepare a thin pancake-like bread with many eyes known as injerra. *Kocho* is a product which is prepared from decorticated and pounded pulp of enset plant (*Ensete ventricosum*), which is further mixed and kneaded into a mash and fermented in a pit.

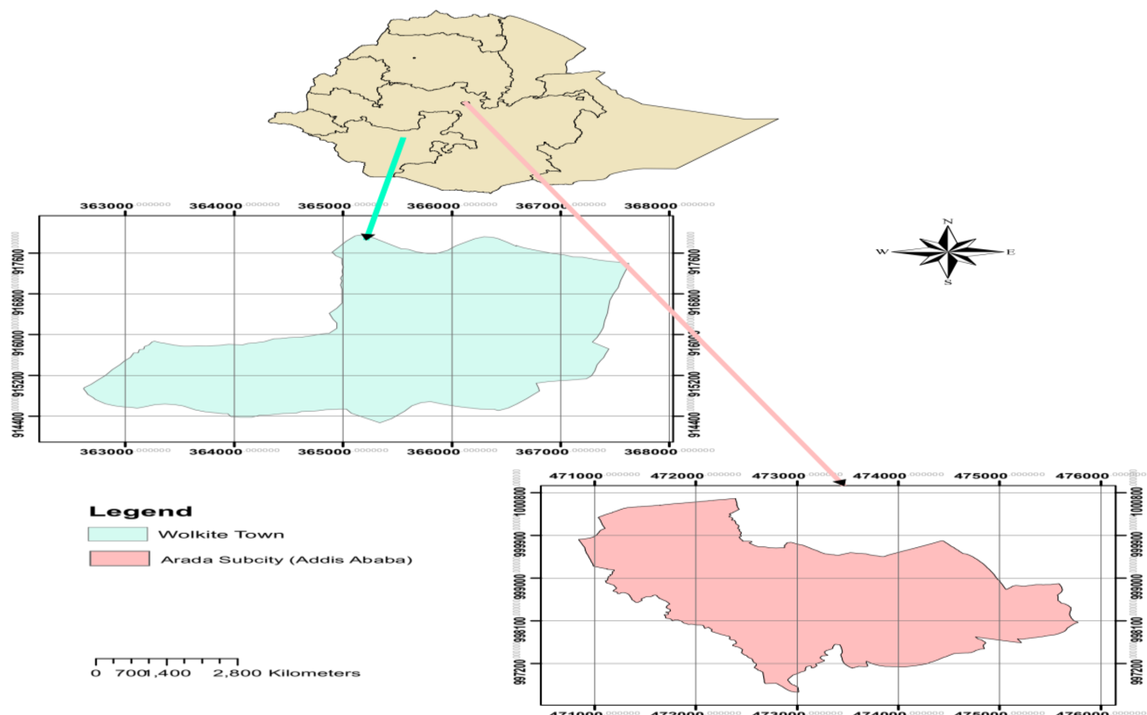

Figure 3.2: Geographic coordinates for fermented food sampling sites

3.2.2 Isolation and Purification of LAB from Traditional Fermented Foods

For isolation of LAB, 25 ml or 25 g of each sample of traditional fermented foods (*teff* dough, *kocho* and *ergo)* was mixed with 225 ml of separate sterile peptone water (0.1% W/V). Then, a sequential decimal dilution of the homogenate was obtained. From the appropriate dilutions, 0.1 ml aliquots were spread plated in duplicates on pre-dried surfaces of de-Man, Rogosa, and Sharp (MRS) agar (Oxoid, Basingstoke, Hampshire, England) plates. The inoculated plates were incubated under anaerobic condition using anaerobic jar (BBL, GasPak Anaerobic Systems) at 37°C for 48 hours. Then, 10-20 distinct colonies were randomly picked from countable MRS plates for purification and further identification. The isolated colonies suspected as LAB were transferred into about 5 ml MRS broth (Oxoid) and purified by repeated streaking on MRS agar. Pure cultures of LAB were then streaked on slants of MRS agar and stored at +4°C for further characterization (Patil *et al.*, 2010).

The LAB isolates were designated by AAUE for *ergo*, AAUT for *teff* dough and AAUK for *kocho*, followed by different numbers.

3.2.3 Confirmation of LAB Isolates

The LAB isolates were further confirmed using the following tests: KOH test, catalase test, spore staining (Appendix 1).

3.2.4 Long Term Preservation of Isolates

Gram-positive, catalase-negative and non-spore forming isolates were preserved in MRS broth medium containing 20% (v/v) glycerol as frozen stocks at -20°C. Glycerol stocks samples were prepared by mixing 0.5 ml of active cultures and 0.5 ml MRS medium including 40% sterile glycerol.

3.2.5 *In Vitro* Characterization of Probiotic Properties

The common methods for *in vitro* analysis of potential probiotic properties include tolerance to low pH, tolerance to bile salt, antibiotic susceptibility, antimicrobial activity, bacteriocin production and bacterial adherence to stainless steel plates.

3.2.5.1 Resistant to Low pH

The determination of the probiotic properties of the isolates was done on the basis of its resistance to low pH. Hence, the isolates were grown separately overnight in 5 ml MRS broth at +37°C under anaerobic conditions. A volume of 1 ml of log 7 CFU/ml of each overnight-grown culture was inoculated into 10 ml of MRS broth to give an initial inoculum level of log 6 CFU/ml. The culture was then centrifuged at 5000 rpm for 10 min at +4°C. The pellets were washed twice in phosphate-saline buffer (PBS at pH 7.2). The pellets were re-suspended in 5 ml sterile MRS broth which was adjusted to pH values of 2.0, 2.5, and 3.0 using 1 N HCl to simulate the gastric environment. The test tubes were incubated for 3 and 6 hours at 37°C. After an appropriate incubation period, 1 ml of the culture was diluted in sterile 9 ml phosphate buffer saline (Sigma, St. Louis, MO USA) prepared according to the manufacturer's instruction (0.1 M, pH 6.2) in order

to neutralize the medium acidity. Briefly, a 100 µl aliquot of the culture and its ten-fold serial dilutions were plated on MRS agar medium. The inoculated plates were incubated at 37°C for 24 to 48 h under anaerobic conditions using anaerobic jar (BBL, Gas Pack System). The grown LAB colonies were expressed in colony-forming units per milliliter (CFU/ml). A positive control consisting of regular MRS broth inoculated with the culture was simultaneously used (Grosu-Tudor and Zamfir, 2012). The survival rate was calculated as the percentage of LAB colonies grown on MRS agar compared to the initial bacterial concentration:

$$\text{Survival rate (\%)} = \frac{\log \text{CFU N1}}{\log \text{CFU N0}} \text{X100}$$

where N_1 is the viable count of isolates after incubation and N_0 is the initial viable count.

3.2.5.2 Tolerance to Bile Salts

To estimate bile tolerance of acid-tolerant probiotic LAB (those only were grown in pH 2.0, 2.5, and/or 3.0), the isolates were separately grown overnight in MRS broth at 37°C under anaerobic conditions (Grosu-Tudor and Zamfir, 2012). Each culture with the initial concentration of 10^6 CFU/ml was then centrifuged at 5000 rpm for 10 min at 4°C. The pellets were washed twice in phosphate-buffer saline (PBS at pH 7.2). Cell pellets were re-suspended in sterile MRS broth supplemented with 0.3% (w/v) bile salt (Oxgall, USA). Samples were taken at 24 h from the onset of incubation to determine the survivability of the cells as described previously (Grosu-Tudor and Zamfir, 2012). A positive control consisting of plain MRS broth without bile salts inoculated with each separate culture was simultaneously incubated. After appropriate incubation, 1 ml of each

separate culture was diluted separately in sterile 9 ml phosphate buffer (Sigma, St. Louis, USA) prepared according to the manufacturer's instruction (0.1 M, pH 6.2) in order to neutralize the medium. Concisely, a 100 µl aliquot of the culture and its ten-fold serial dilutions were plated on MRS agar medium. Plates were incubated at 37°C for 24 to 48 h under anaerobic conditions using anaerobic jar (BBL, Gas Pack System). LAB counts were expressed in colony-forming units per milliliter (CFU/ml). The survival rate was calculated as the percentage of LAB colonies grown on MRS agar compared to the initial bacterial concentration:

$$\text{Survival rate (\%)} = \frac{\log \text{CFU N1}}{\log \text{CFU N0}} \text{X100},$$

where N_1 is the viable count of isolates after incubation and N_0 the initial viable count.

3.2.5.3 Antimicrobial Activity

Antibacterial activity of the acid-bile-tolerant probiotic LAB strains against some foodborne pathogens was determined using the agar well diffusion method with some modifications of the protocol indicated by Fontana *et al.* (2015). The test organisms (*Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* (clinical isolate), *Salmonella* Typhimurium and *Escherichia coli* ATCC 25922 were obtained from Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia.

The selected acid-bile-tolerant LAB isolates were inoculated from slants to fresh MRS broth containing 1% glucose and incubated overnight at 37°C. The overnight active culture broth of each isolate was centrifuged separately at 5000 rpm for 10 min at 4°C. The cell-free supernatant from each separate culture was collected as a crude extract for

the antagonistic study against selected foodborne pathogens. The pure cultures of foodborne pathogens were inoculated from slants to brain heart infusion broth. After 24-hour incubation at 37°C, a volume of 100 µl of inoculum of each standard bacterium was swabbed evenly over the surface of nutrient agar plates with a sterile cotton swab. The plates were allowed to dry, and a sterile cork borer (diameter 5 mm) was used to cut uniform wells in the agar. Each well was filled with 100 µl culture-free filtrate obtained from each of the acid-bile-tolerant LAB isolates. After incubation at 37°C for 24 to 48 hours, the plates were observed for a zone of inhibition (ZOI) around the well. The diameter of the ZOI was measured by calipers in millimeters, and a clear zone of 2 mm or more was considered positive inhibition (del Mar Lleo *et al.*, 1998). The experiment was carried out in triplicates, and the activity was reported as the diameter of ZOI±SD.

3.2.5.4 Production of Bacteriocins

The screened 34 LAB isolates were tested for their ability to produce bacteriocins. Hence, the presence of bacteriocins in the probiotic LAB isolates were qualitatively determined by agar well diffusion assay method against *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* (clinical isolate), *Salmonella* Typhimurium and *Escherichia coli* ATCC 25922 (Yang *et al.*, 2012).

The selected LAB isolates were grown individually in MRS broth for 16 to 18 h at 37°C. The overnight active culture broth of each isolate was centrifuged separately at 5000 rpm for 10 min at 4°C. The supernatants obtained were adjusted to pH 6.5 with 1 N NaOH in order to rule out possible inhibition effects due to organic acids. To avoid proteolytic degradation of the bacteriocin, cell-free supernatant (CFS) was heated in a water bath at

100°C for 5 min (Simova *et al.*, 2009). Petri dishes with 20 ml of semisolid brain heart infusion (BHI; Himedia, India) were prepared and a volume of 0.1 ml active culture of each test organism was spread plated on pre-dried BHI agar. After solidification, wells were made and aliquots of 50 µl of CFS from each LAB isolate were added to each well. The BHI plates were incubated at 37°C aerobically for 24 h. Inhibition zones were measured using an electronic caliper with digital display (Master craft MD, Miami, FL, USA). The experiment was carried out in triplicates and activity was reported as the diameter of ZOI ± SD. Inhibitory reaction scored positive if the width of a clear zone around the colonies of LAB isolates was 2 mm or larger.

To confirm the production of bacteriocin, CFS displaying antimicrobial potential after acid neutralization and heat treatment were tested with proteinase K (Sigma, St. Louis, USA). Therefore, a volume of 3 µl of proteinase K was added to each test tube containing 5 ml of CFS. The test tubes with and without the enzyme (control) were incubated at 37°C for 2 hours. Both control and treated samples were assayed for antimicrobial activity by using the agar well diffusion method as described by (Saad *et al.*, 2015).

3.2.5.5 Antibiotic Susceptibility Tests

Each of acid-bile-tolerant and antagonistic lactic acid bacterial isolates were assessed for their antibiotic resistance by the disc diffusion method as described by Zhang *et al.* (2016) using some antibiotics that included ampicillin (10 µg/ml), erythromycin (15 µg/ml), streptomycin (10 µg/ml), kanamycin (25 µg/ml) and tetracycline (30 µg/ml). Thus, a volume of 100 µl of actively growing cultures of each acid-bile-tolerant and antagonistic lactic acid bacteria was swabbed evenly over the surface of nutrient agar

plates with a sterile cotton swab. After drying, the antibiotic discs were placed on the solidified agar surface, and the plates were left over for 30 min at 4% for diffusion of antibiotics and then anaerobically incubated at 37°C for 24 to 48 h. Resistance was defined according to the disc diffusion method by using the above antibiotic discs and the diameters of inhibition zones were measured using calipers (Vlková *et al.*, 2006). The zone of inhibition (diameter in mm) for each antibiotic was measured and expressed as susceptible, S (≥21 mm); intermediate, I (16-20 mm) and resistance R (≤15 mm).

3.2.5.6 Bacterial Adhesion to Stainless Steel Plates

The adherence assay of acid-bile-tolerant, antagonistic and antibiotic-sensitive probiotic lactic acid bacterial isolates was determined on stainless steel plates with some modifications method of El-Jeni *et al.* (2015). Briefly, from the overnight bacterial culture in MRS broth, 500 µl was deposited in a test tube, which was then filled with 450 µl of MRS broth, wherein the sterile stainless steel plate was deposited, and the test tubes were then incubated for 24 h at 37°C. The stainless steel plate was removed under aseptic conditions, washed with 10 ml of sterile 1% peptone water, and left for 5 min in a sterile 1% peptone water tube. The plate was then washed again in the same conditions and vortexed for 3 min in a sterile 1% peptone water tube (6 ml) consecutively to detach the bacterial cells adhering to the steel plate surface. LAB counts were expressed in colony-forming units per milliliter (CFU/ml). A positive control inoculated with culture was simultaneously used. The adherence rate was calculated as the percentage of LAB colonies grown on MRS agar compared to the initial bacterial concentration.

3.2.6 Morphological, Biochemical and Physiological Tests

The LAB isolates were identified according to their morphological, physiological and biochemical characteristics based on Bergey's Manual (Nair and Surendran, 2005).

Overnight cultures were carried out for their gram staining technique and examined under a light microscope using oil immersion objectives. Cellular morphological criteria considered during examination were cell shape and cell arrangement.

3.2.6.1 Growth at Different Temperatures

Each of the overnight LAB cultures of 50 µl was transferred into separate tubes that contained 5 ml medium (modified MRS broth) containing bromoecresol purple indicator at a concentration of 0.12 g/l. After inoculation, they were incubated for 7 days at 15ºC and 45ºC. During this incubation time, growth at any temperature was observed by the change of the cultures, from purple to yellow (Yavuzdurmaz, 2007).

3.2.6.2 Growth at Different NaCl Concentrations

LAB isolates were tested for their tolerance to different NaCl concentrations. For this purpose, 4% and 6.5% NaCl concentrations were used for testing. Similarly, test tubes with 5 ml of modified MRS broths containing bromoecresol purple indicator. Then, 34 test tubes with 4% NaCl and the other 34 test tubes with 6.5% NaCl were inoculated separately with 50 µl of 1% of each overnight culture of LAB and incubated at +37°C for 7 days. The change of the color from purple to yellow was considered as a proof of the cell growth (Yavuzdurmaz, 2007).

3.2.6.3 Arginine Hydrolysis Test

Arginine containing MRS medium and Nessler's reagent (HgI_4K_2), a 0.09 mol/L solution of potassium tetraiodomercurate (II) (K_2 [HgI_4]) in 2.5 mol/L potassium hydroxide, were used in order to see ammonia production from arginine. MRS containing 0.3% L-arginine hydrochloride was transferred into duplicate tubes with 5 ml amount and inoculated with each of 1% overnight cultures of LAB. Thereafter, tubes were incubated at +37°C for 24 h. A 100 µl of cultures were transferred onto a white background. Thereafter, the same amount of Nessler's reagent was pipetted to the cultures. The change in color to bright orange indicates a positive reaction, while yellow color indicates the negative reaction. A MRS medium, which did not contain arginine, was used as negative control (Rodríguez *et al.*, 2000).

3.2.6.4 Gas Production from Glucose

In order to determine the homofermentative and heterofermentative characteristics of LAB isolates, CO_2 production from glucose was determined in modified MRS broth containing inverted Durham tubes with 1% glucose. MRS broth (8 ml) in separate tubes containing 1% glucose with inverted Durham tubes was prepared and inoculated separately with 50 µl of 1% overnight fresh LAB cultures. Then the test tubes were incubated at +37°C for 5 days. The presence of gas in Durham tubes during 5 days of observation indicates CO_2 production from glucose (Bulut, 2003).

3.2.7 Molecular Characterization of Probiotic Lactic Acid Bacteria

3.2.7.1 Identification of Probiotic LAB isolates by 16S rRNA Gene Sequencing

3.2.7.1.1 Genomic DNA extraction

Genomic DNA was extracted from pure cultures (n = 4) of potential probiotic LAB. 1 ml of each pure liquid culture was centrifuged for 3 min at 10,000 rpm. The supernatant was removed, and the cells were re-suspended in 300 µl buffer (10 mM Tris-HCl, pH 8.0; 50 mM glucose, and 10 mM EDTA). To the suspension, 3 µl lysozyme (10 mg/ml) was added, and cells were incubated at 37°C for 60 min under occasional stirring of the tube content by overturning it. A 300 µl of lysis buffer (20 mM Tris-HCl, pH 8.0; 75 mM NaCl; 1% SDS; 10 mM EDTA) and 3 µl RNAse (10 mg/ml) were added to the mixture. The mixture was incubated at 37°C for 30 min and then cooled on ice for 1 min. Then, 100 µl of ammonium acetate (7.5 M) was added to the mixture, mixed on a vortex for 20 seconds and was centrifuged at 13,000 rpm for 5 min. The supernatant was transferred into clean 1.5 ml tubes, and 300 µl isopropanol was added. Thereafter, the mixture was mixed by overturning for 1 min and stored at –20°C for 30 min. The mixture was centrifuged at 13,000 rpm for 5 min. The supernatant was accurately decanted, and the tubes were placed overturned on a clean filter. Then, 400 µl of 70% ethanol was added and mixed several times by overturning to wash the DNA sediment. Finally, the sediment was dried at 37°C for 15 min till ethanol drops disappeared. The dried sediment was dissolved in 30 µl TE buffer.

3.2.7.1.2 PCR Amplification of 16S rRNA

For the amplification of the 16S rRNA gene, the specific primers AMP_F 5′- GAG AGT TTG ATY CTG GCT CAG -3′ and AMP_R 5′- AAG GAG GTG ATC CAR CCG CA -3′ were used. PCR reaction mixture was prepared by mixing 25 µl of the Tag 2x Master mix (buffer, polymerase, and dNTPs), forward primer 1 µl, reverse primer 1 µl and UPH2O 22 µl. Then, 49 µl of the mixtures were added to a sterile PCR tube, and 1 µl of the gDNA was used as a template; the amplification reactions were carried out in a thermal cycler (Bio-Rad Mycycler). The PCR program used was:

Step 1: 95°C for 2 min
Step 2: 95°C for 30 s
Step 3: 55°C for 30 s — 25 cycles
Step 4: 72°C for 1 min
Step 5: 72°C for 5 min

3.2.7.1.3 DNA Electrophoresis

PCR products were separated in a 1% agarose gel in 0.5xTris/Borate/EDTA (TBE) buffer at 90V for 1 h. The gel was stained with ethidium bromide followed by examination under a UV illuminator and images were captured by a digital camera.

3.2.7.1.4 Sequencing of the PCR Products

16S rRNA PCR products were purified using the QIAquick PCR Purification Kit (Quagen, Germany) and sequencing was performed by Eurofins (Germany).

3.2.7.1.5 Phylogenetic Analysis

Forward and reverse sequences were assembled and edited using BioEdit Sequence Alignment Editor Version 5.0.9. Sequence similarity was estimated by searching the homology in the Genbank DNA database using BLAST. Finally, the isolates were then identified based upon the sequence.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.19972900 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 891 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016).

3.2.8.2 Whole Genome Sequencing (WGS) of probiotic LAB Isolates

DNA Extraction

Overnight culture was used to prepare new stocks. Therefore, 0.5 ml of the culture and 0.5 ml of 40% glycerol were mixed in a sterile 1.5 ml tube and placed at -80°C. They were inoculated at 2% and incubated at 37°C until OD=1. The culture was centrifuged at 4000 rpm 15 min 4°C and the supernatant was discarded. The pellet was resuspended in 1

ml 10 mM Tris-HCL pH 8.5 Buffer (EB buffer) and all the mixture transferred to 3x1.7 ml tubes. The tubes were centrifuged at maximum speed for 2 mins and the supernatant was discarded. Pellets were washed with 400 µl of colony wash buffer (100 mM NaCl, 10 mM Tris pH7, 1 mM EDTA) and the supernatant was removed with pipette and pellets were stored at -20°C. Pellets were re-suspended in 400 µl of EB buffer and 20 µl 100 mg/ml lysozyme, 5 µl mutanolysin (10U/ µl), 45 µl 20 mg/ml proteinase K and 1 µl 10 mg/ml RNAse were added and incubated at 37°C for 15-30 min. Then, 70 µl 10% SDS was added and mixed by inversion, and incubated at 65°C for 10 mins on heat block. Following this, 100 µl 5 M NaCl was added, mixed and then 100 µl of the preheated CTAB (10% IN 0.7 M NaCl) was added. The mixture was homogenized and incubated for 10 mins at 65°C on a heat block. Afterward, 500 µl chloroform: isoamyl alcohol (24:1) were added and mixed for at least 10 seconds and centrifuged at maximum speed for 5 mins. There were three layers formed- a clear upper layer, a cloudy middle layer, and a clear lower layer. Hence, the upper layer was transferred carefully to a new 1.7 ml tube and 0.6x volumes of isopropanol were added (i.e. If there is 500 µl of the clear layer, 300 µl isopropanol was added (0.6x500)), mixed and incubated at -20°C for at least 30 mins, and then centrifuged at maximum speed for 10 mins. The supernatant was discarded and the pellets were washed with 500 µl cold 70% ethanol. The pellets were air-dried for 5 mins and re-suspended in 50 µl EB buffer. One µl 10 mg/ml RNAse was added and incubated at 37°C for 30 mins on the heat block. Five µl 3 M NaAcetate pH 8.0 was added to the sample carefully along the side of the test tube. In the same way, 100 µl ice-cold 99% ethanol was added to the tube. The tube was mixed by flicking gently. Then the tubes were centrifuged at the maximum speed for 2 mins, the

supernatant was discarded with a pipette and the pellet washed with 70 µl of 70% cold ethanol. The supernatant was removed with a pipette, air-dried for 5 mins and finally, the DNA was re-suspended in 50 µl of EB buffer. A 1 µl of DNA was visualized on 1% agarose gel according to the protocol in section 3.2.8.1.3., DNA concentration was measured using Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific) and DNA quality was assessed by measuring the 260/230 and 260/290 absorbance ratio using nanodrop.

3.2.8.3 Whole Genome Sequencing

DNA extracted following the protocol in section 3.2.8.2 was used to obtain whole-genome sequences in the Earlham Institute (Norwich, UK). Briefly, Low Input Transposase Enabled (LITE) libraries were constructed and sequenced using an Illumina HiSeq 4000 platform. Raw reads were processed as follows: adapters were removed from 3'-end, bases with quality less than phred 3 were removed from both ends and reads with a length below 100 bp or average quality of less than phred 20 were discarded using bbduk. Then, cleaned read sets were normalized to 2x-100x coverage and assembled *de novo* using SPAdes (version 3.8.1). Finally, the genome sequences were annotated using RAST pipeline (http://bioinf.spbau.ru/en/spades and http://rast.nmpdr.org/).

3.2.8.4 Bacteriocin Prediction

To predict putative bacteriocins, BAGEL4 software was used. Therefore, a blast (blastx) of the contigs sequences was run against the bacteriocin protein sequences (Class I, Class II and Class III) downloaded from the BAGEL4, a web-based bacteriocin genome mining tool database (http://bagel4.molgenrug.nl/databases.php). All contigs sharing with these

bacteriocin proteins matches with a P-value lower than e-10 and a sequence identity higher than 80% was considered as containing a bacteriocin protein.

3.3 Data Analysis

All the measurements were performed in triplicate, and results were expressed as mean standard deviation (SD). Data were analyzed by SAS software (ver. 9.2, Raleigh, NC). The phylogenetic tree was prepared using MEGA7 (version 7.0). Statistical significance was determined at $p<0.05$.

3.4 Results

3.4.1 Isolation of Potentially Probiotic LAB from Traditional Fermented Foods

A total of 45 samples (15 samples from each) of the three traditionally fermented Ethiopian foods products were used for isolation of LAB. A total of 450 (150 from each sample) probiotic LAB were isolated from three different traditionally fermented Ethiopian food products at 37°C under anaerobic conditions. Out of the total 450 probiotic lactic acid bacteria isolates, 260 (57.78%) isolates were found Gram-positive, endospore negative and catalase-negative. From the total 260 presumptive probiotic lactic acid bacterial isolates, 101 (38.85%), 81 (31.15%) and 78 (30%) were from *teff* dough, *ergo* and *kocho*, respectively.

3.4.2 Morphological, Biochemical and Physiological Characterization

The 34 acid-bile-tolerant potential probiotic LAB isolates were identified through their morphological, biochemical and physiological features (Table 3.4). Among them, 28 (82.35%) LAB isolates were straight rod-shaped. However, the remaining 6 (17.65%) probiotic isolates were spherical shaped. All the 34 acid-bile-tolerant LAB isolates were able to grow at 4% NaCl salt concentration. From the 34 acid-bile-tolerant probiotic LAB isolates, 30 (88.24%) of the isolates grew at 6.5% NaCl salt concentration. On the contrary, 4 (11.26%) isolates did not grow at 6.5% NaCl salt concentration. Another criterion for the identification of the isolates was their ability to grow at different temperatures. Hence, all the 34 acid-bile-tolerant LAB isolates were able to grow at

15°C. Among them, 28 (82.35) LAB isolates were able to grow at 45°C, whereas, 6 (17.65%) isolates were not found growing at 45°C (Table 3.4).

Out of the 34 acid-bile-tolerant isolates, 9 (26.47%) isolates produced gas from glucose (Table 3.4). Whereas the majority of LAB isolates, 25 (73.53%), did not produce gas from glucose. Regarding the arginine hydrolysis test, among the 34 acid-bile-tolerant isolates, 14 (41.18%) potential probiotic LAB isolates were found positive for arginine hydrolysis, whereas 20 (58.82%) isolates did not hydrolyze arginine (Table 3.4).

Among the 34 acid-bile-tolerant isolates, 11 isolates (32.35%) were obtained from *ergo*, 10 (29.41%) isolates were obtained from *kocho* and the remaining 13 (38.24%) isolates were obtained from *teff* dough (Table 3.4).

Table 3.4: Physiological, morphological and biochemical characteristics of probiotic LAB isolates

Isolate	**KOH test**	**Catalase test**	**Shape**	**Spore staining**	**Temperature**		**Salt concentration**		**Production**	
					15°C	45°C	4%	6.5%	CO_2	NH_3
AAUE143	-ve	-ve	Cocci	-	+	-	+	+	-	-
AAUT126	-ve	-ve	Rod	-	+	+	+	+	-	+
AAUT064	-ve	-ve	Rod	-	+	+	+	+	-	-
AAUT136	-ve	-ve	Rod	-	+	+	+	+	-	-
AAUE132	-ve	-ve	Rod	-	+	+	+	+	-	-
AAUT134	-ve	-ve	Rod	-	+	+	+	+	-	-
AAUT115	-ve	-ve	Rod	-	+	+	+	+	-	+
AAUT075	-ve	-ve	Rod	-	+	+	+	+	-	-
AAUK070	-ve	-ve	Rod	-	+	-	+	+	-	-
AAUT082	-ve	-ve	Rod	-	+	+	+	+	-	-
AAUT081	-ve	-ve	Rod	-	+	+	+	+	+	-
AAUT101	-ve	-ve	Rod	-	+	+	+	+	-	-
AAUT104	-ve	-ve	Rod	-	+	+	+	+	-	-
AAUT121	-ve	-ve	Rod	-	+	+	+	+	-	-
AAUE116	-ve	-ve	Cocci	-	+	+	+	+	-	+
AAUE063	-ve	-ve	Cocci	-	+	-	+	-	-	+
AAUE124	-ve	-ve	Cocci	-	+	+	+	+	-	+

AAUK040	-ve	-ve	Rod	-	+	+	+	+	-	-
AAUK135	-ve	-ve	Rod	-	+	+	+	+	-	-
AAUK132	-ve	-ve	Rod	-	+	+	+	+	+	+
AAUE144	-ve	-ve	Cocci	-	+	+	+	+	-	-
AAUE051	-ve	-ve	Rod	-	+	-	+	-	+	+
AAUE048	-ve	-ve	Rod	-	+	+	+	+	-	-
AAUE142	-ve	-ve	Cocci	-	+	-	+	-	-	+
AAUK114	-ve	-ve	Rod	-	+	+	+	+	+	+
AAUE052	-ve	-ve	Rod	-	+	+	+	-	-	-
AAUK122	-ve	-ve	Rod	-	+	+	+	+	-	-
AAUK071	-ve	-ve	Rod	-	+	+	+	+	+	+
AAUK072	-ve	-ve	Rod	-	+	+	+	+	-	-
AAUK082	-ve	-ve	Rod	-	+	+	+	+	+	+
AAUT146	-ve	-ve	Rod	-	+	+	+	+	+	-
AAUE031	-ve	-ve	Rod	-	+	-	+	+	-	+
AAUT035	-ve	-ve	Rod	-	+	+	+	+	+	+
AAUK011	-ve	-ve	Rod	-	+	+	+	+	+	+

Legend: + = positive result, - = negative result

3.4.3 *In Vitro* Characterization of Probiotic Properties

3.4.3.1 Resistance to Low pH

Out of 260 probiotic LAB isolates, 34 (13.10%), 34 (13.10%), and 65 (25.00%) isolates tolerated pH values of 2, 2.5, and 3 for 3 h, respectively (Table 3.5). Upon further extension of the incubation period to 6 h, the 68 lactic acid bacteria isolates (each 34 tested at pH 2.0 and 2.5) survived, whereas only 41 survived from 65 with the extension of incubation period to 6 h at pH 3.0 (Table 3.5).

Accordingly, out of the total 260 LAB isolates, 34 (13.10%) isolates survived pH 2.0, 2.5, and 3.0 upon exposure for 3 and 6 hours, and the mean value of the treatments was significantly different at $p<0.05$ (Table 3.5). Among them, 13 (38.24%), 11 (32.35%) and 10 (29.41%) probiotic lactic acid bacteria were isolated from *teff* dough, *ergo* and *kocho* samples, respectively (Table 3.5).

Table 3.5**:** Acid tolerance patterns of LAB at different pH values after 3 and 6 h exposure

Source	**No. of isolates**	**Number of survived isolates (%)**					
		3 h			**6 h**		
		pH 2	**pH 2.5**	**pH 3**	**pH 2**	**pH 2.5**	**pH 3**
Teff dough	101	13 (12.87%)	13 (12.87%)	25 (24.75%)	13 (12.87%)	13 (12.87%)	16 (15.84%)
Ergo	81	11 (13.58%)	11 (13.58%)	21 (25.93%)	11 (13.58%)	11 (13.58%)	13 (16.10%)
Kocho	78	10 (12.82%)	10 (12.82%)	19 (24.36%)	10 (12.82%)	10 (12.82%)	12 (15.38%)
Total	260	34 (13.10%)	34 (13.10%)	65 (25.00%)	34 (13.10%)	34 (13.10%)	41 (15.77%)

In general, the survival rate of the LAB isolates ranged from 33.33 to 99.32% at different pH values upon incubation for 3 and 6 h period and they were significantly different at p<0.05 (Table 3.6). Among the isolates exposed to pH 2 for 3 h, isolate AAUE124 was the most tolerant with a 96.33% survival rate, followed by isolates AAUK114 and AAUK132 with 95.59% and 93.7% survival rate, respectively. However, isolate AAUE031 was the least tolerant with a 45.35% survival rate. Similarly, the same isolates, AAUK114, AAUE124 and AAUK132 were the most tolerant with 91.75%, 90.61%, and 89.35% survival rate, respectively at pH 2 for 6 h. Although the majority of the isolates were tolerant to pH 2.0 for 6 h, only 11 (32.35%) isolates were grown below 50% ranged from 33.33 to 48.68% (Table 3.6).

This is similar to isolates AAUE124, AAUK114, AAUK132 and AAUT115 which were found highly tolerant to pH 2.5 and 3.0 for 3 h with survival rates ranging from 95.40 to 99.32% (Table 3.6). On the other hand, isolate AAUT136 was found the least tolerant to pH 2.5 and 3.0 for 3 h with survival rates 64.54% and 75.17%, respectively. Though almost all the isolates survived to the specified acidic condition, the survival percentage of the isolates was significantly reduced at lower pH than higher pH as well as time exposure. Regarding to pH 2.5 for 6 h, the survival rate of the LAB isolates ranged from 50.40 to 95.53% (Table 3.6). Whereas, with respect to pH 3 for the 6 h incubation period, the survival rate of the LAB isolates ranged from 61.22 to 98.17%. Isolates AAUE124 was the most tolerant with a 95.53% survival rate at pH 2.5 for 6 h, followed by isolate AAUK114 with a 93.56% survival rate. Likewise, out of the selected 34 LAB isolates exposed to pH 3 for 6 h, isolate AAUE124 was the most tolerant with 98.17% survival rate, followed by isolate AAUK132 and AAUK114 with 96.68% and 96.38% survival rate, respectively (Table 3.6).

Table 3.6**:** Percentage survival of probiotic LAB at different pH levels and 0.3% bile salt

Isolates	**pH tolerance**						**Bile tolerance**
	3 h			**6 h**			**24 h**
	pH 2	**pH 2.5**	**pH 3**	**pH 2**	**pH 2.5**	**pH 3**	**0.3%**
AAUE143	62.43±1.28^{ghijk}	74.67±1.28^{kl}	85.70±1.83^{l}	50.30±1.11^{efg}	65.09±1.31^{ij}	80.12±1.11^{mn}	92.54±2.72^{ghijkl}
AAUT126	60.60±1.39^{jkl}	77.55±1.55^{hij}	90.27±0.86^{ghijk}	52.81±1.05^{e}	64.15±1.05^{jk}	80.18±1.21^{mn}	94.67±1.29^{defghi}
AAUT064	61.29±1.23^{hijkl}	78.71±1.07^{fghi}	89.24±1.07^{jk}	51.70±1.42^{ef}	69.47±1.05^{gh}	82.46±0.70^{kl}	90.77±2.53^{klm}
AAUT136	54.45±1.40^{n}	64.54±1.44^{o}	75.17±1.61^{o}	44.09±1.22^{j}	53.78±1.83^{n}	61.22±1.40^{s}	83.88±4.20^{qr}
AAUE132	50.52±0.70^{p}	77.70±1.39^{hij}	91.87±2.47^{defghi}	46.69±1.26^{hi}	74.80±1.57^{de}	84.90±1.92^{hij}	95.94±2.21^{bcdef}
AAUT134	51.95±0.98^{op}	68.39±3.02^{mn}	81.06±1.78^{n}	35.52±1.43^{l}	51.17±0.94^{o}	71.99±1.65^{q}	84.98±1.38^{pqr}
AAUT115	90.23±1.44^{c}	95.40±0.72^{b}	98.62±1.03^{a}	82.99±0.72^{b}	90.58±1.31^{b}	94.83±0.69^{b}	98.16±1.44^{abc}
AAUT075	53.66±1.05^{no}	69.45±0.73^{m}	81.88±0.70^{mn}	39.84±1.06^{k}	51.57±0.92^{no}	71.78±1.05^{q}	82.58±1.40^{r}
AAUK070	50.52±0.69^{p}	74.91±1.03^{lk}	92.78±1.03^{cdefg}	33.33±1.03^{l}	50.40±1.43^{o}	82.70±0.72^{jk}	95.72±1.19^{bcdefg}
AAUT082	56.67±1.70^{m}	69.01±1.54^{mn}	83.08±1.13^{mn}	38.15±2.06^{k}	53.58±1.90^{n}	72.34±0.77^{q}	86.66±0.53^{opq}
AAUT081	62.48±0.77^{ghijk}	80.81±0.74^{ef}	92.74±0.56^{cdefg}	53.01±1.13^{e}	71.46±1.13^{fg}	85.36±0.56^{ghi}	93.36±2.29^{efghijlk}
AAUT101	59.67±0.53^{l}	73.90±0.53^{l}	92.19±1.07^{defghi}	52.92±0.53^{e}	65.38±0.70^{ij}	78.20±1.07^{mnop}	93.02±2.03^{fghijk}
AAUT104	63.33±0.90^{ghi}	79.29±0.94^{fgh}	94.17±1.09^{cd}	51.90±1.44^{ef}	59.40±1.09^{m}	80.36±0.71^{lm}	95.06±1.05^{cdefgh}
AAUT121	72.22±0.74^{e}	86.18±1.40^{d}	93.83±1.30^{cde}	52.84±0.57^{e}	76.92±1.13^{cd}	90.25±1.13^{cd}	93.44±1.39^{efghijk}
AAUE116	62.62±1.80^{ghij}	81.90±1.44^{e}	95.24±2.09^{cb}	50.60±1.69^{efg}	62.26±1.83^{kl}	89.64±1.64^{cde}	96.35±1.43^{abcde}
AAUE063	62.83±1.50^{ghij}	76.38±1.10^{ijk}	89.69±1.45^{ijk}	52.76±1.26^{e}	61.99±1.10^{kl}	78.18±0.91^{mnop}	94.25±1.74^{defghij}
AAUE124	96.33±0.86^{a}	97.94±0.69^{a}	99.20±0.52^{a}	90.61±1.21^{a}	95.53±1.37^{a}	98.17±1.05^{a}	99.44±0.39^{a}

AAUK040	55.48±1.41^{mn}	70.16±0.73^{m}	83.69±0.73^{lm}	45.80±1.26^{ij}	53.97±1.07^{n}	63.99±0.70^{r}	88.96±1.18^{mno}
AAUK135	74.05±1.59^{de}	86.74±0.72^{d}	97.35±1.40^{ab}	62.40±1.22^{c}	79.01±1.06^{c}	90.31±1.59^{cd}	98.10±1.18^{abc}
AAUK132	93.70±0.86^{b}	96.22±1.03^{ab}	98.97±0.91^{a}	89.35±1.03^{a}	91.07±1.37^{b}	96.68±1.21^{ab}	98.88±1.08^{ab}
AAUE144	61.00±1.39^{ijkl}	73.49±1.36^{l}	90.86±1.94^{fghij}	50.97±1.16^{efg}	67.06±1.56^{hi}	78.63±1.82^{mno}	91.72±2.59^{ijklm}
AAUE051	61·78±1.05^{hijkl}	75.87±1.47^{jkl}	89.96±0.84^{ijk}	52.72±1.51^{e}	66.39±1.74^{ij}	76.15±1.51^{p}	87.57±1.77^{nop}
AAUE048	62.14±0.95^{hijk}	80.76±1.36^{ef}	93.21±1.00^{cdef}	52.20±1.33^{e}	66.04± 1.13^{ij}	86.92± 1.33^{fgh}	94.07±2.07^{defghij}
AAUE142	63.70± 3.82^{gh}	70.74± 2.41^{m}	90.14± 0.94^{hijk}	52.89± 3.02^{e}	69.48± 1.41^{gh}	84.67± 1.18^{hijk}	98.65±0.89^{ab}
AAUK114	95.60±1.55^{ab}	97.86±0.52^{a}	99.32±0.68^{a}	91.75±0.71^{a}	93.56±1.55^{a}	96.38±1.04^{ab}	99.34±0.33^{a}
AAUE052	57.36± 1.63^{m}	80.31± 1.17^{efg}	93.02± 1.40^{cdef}	48.68± 1.63^{gh}	65.58± 1.68^{ij}	88.68± 1.88^{cdef}	93.62±2.19^{efghijk}
AAUK122	54.26±0.78^{n}	67.05±1.55^{n}	88.24±1.18^{k}	45.22±1.36^{ij}	60.85±1.78^{lm}	76.49±1.57^{op}	89.72±1.33^{lmn}
AAUK071	64.82±2.01^{g}	77.72±1.76^{hij}	91.46±1.81^{efghij}	51.42±3.07^{efg}	69.01±1.26^{gh}	87.44±2.30^{efg}	94.74±0.48^{defghi}
AAUK072	62.42±1.66^{ghijk}	76.42±1.42^{ijk}	89.94±0.98^{ijk}	51.26±2.13^{efg}	64.94±1.19^{ij}	77.83±1.89^{nop}	92.34±1.62^{hijkl}
AAUK082	69.92±1.56^{f}	79.56±1.19^{efgh}	92.58±0.78^{defgh}	51.43±1.37$_{efg}$	67.06±1.19^{hi}	79.30±0.78^{mn}	92.58±1.17^{ghijkl}
AAUT146	74.97±1.10^{d}	88.21±1.10^{cd}	98.19±0.96^{a}	55.84±0.75^{d}	73.53±1.10^{ef}	87.97±1.10^{def}	98.38±0.82^{ab}
AAUE031	45.35± 1.08^{q}	79.39± 1.59^{fgh}	94.00± 1.62^{cde}	38.40± 1.24^{k}	71.50± 1.08^{fg}	83.39± 1.27^{ijk}	97.22±0.35^{abcd}
AAUT035	60.15± 0.74^{kl}	77.98± 1.19^{ghij}	90.28± 2.03^{ghijk}	51.41± 0.77^{efg}	70.11± 1.11^{g}	83.89± 0.93^{ijk}	91.37±2.66^{jklm}
AAUK011	73.29± 1.44^{de}	90.13± 1.10^{c}	97.11± 2.17^{ab}	49.34± 1.27^{fg}	70.52± 1.99^{g}	90.49± 1.46^{c}	93.38±0.70^{efghijk}

$^{a-s}$Means not connected by the same letter within a column are significantly different at $p < 0.05$. Results expressed as average ($n = 3$) ± SD (standard deviation).

3.4.3.2 Tolerance to Bile Salts

The probiotic lactic acid bacteria isolates, resistant to low pH, were screened for their ability to tolerate 0.3% bile salt concentration. According to the results, all of the 34 probiotic LAB isolates were able to survive above 82 % in the presence of 0.3% of bile salt concentration and they were significantly ($p<0.05$) different (Table 3.6). In general, the survival rate of the probiotic LAB isolates ranged from 82.58 to 99.44%. Among the selected LAB isolates, isolate AAUE124 was the most tolerant with 99.44% survival rate followed by isolates AAUK114 and AAUK132 with 99.34% and 98.88% survival rate, respectively. On the contrary, isolate AAUT075 showed the least (82.58%) survival rate (Table 3.6).

Accordingly, as all the LAB isolates exhibited overall high resistance to low pH (2.0, 2.5 and 3.0 and 0.3% bile salt concentration and a significantly low death rate, these would be promising probiotic candidates and were taken for the next step.

3.4.3.3 Antimicrobial Activities

The diameter of inhibition zones showed that crude extracts from each isolate had an antimicrobial effect against each tested foodborne pathogen and were significantly ($p<0.05$) different (Table 3.7). The average zones of inhibition by which the crude extracts inhibited the growth of the test foodborne pathogens ranged between 15 to 22 mm. Isolate AAUK114 displayed the highest antagonistic activity against *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes*, *Escherichia coli* ATCC 25922 and *Salmonella* Typhimurium with the inhibition zone ranged from 20.33 to 22 mm in

diameters. However, AAUK082 showed a minimum inhibition zone of diameter ranged from 15 to 17.33 mm against the standard pathogens (Table 3.7).

Specifically, out of the 34 potential probiotic LAB candidates, isolate AAUK122 had an inhibition zone with a minimum diameter of 15.33 mm against *Listeria monocytogenes* and AAUT101 and AAUK114 showed the highest inhibition zone with a diameter of 21 mm against *Listeria monocytogenes* (Table 3.7). Likewise, AAUK070 and AAUK072 showed minimum inhibition zone with a diameter of 16 mm and 16.33 mm and isolate AAUK114 showed maximum inhibition zone with a diameter of 22 mm against *Staphylococcus aureus* ATCC 25923. Isolate K082 showed the lowest inhibition zone with a diameter of 15 mm against *Escherichia coli* ATCC 25922 and *Salmonella* Typhimurium but isolate AAUK011 showed maximum inhibition zone with a diameter of 20.67 mm against *Escherichia coli* ATCC 25922. In addition, isolate AAUT101 and AAUK114 showed the highest inhibition zone with a diameter of 20.67 mm against *Salmonella* Typhimurium (Table 3.7).

Table 3.7**:** Antimicrobial activities of LAB against some foodborne pathogens

Number	Isolates	Diameter of inhibition zone (mm)			
		L. monocytogenes	***S. aureus***	***E.coli***	***S.* Typhimurium**
1.	AAUE143	20.00±1.00^{abc}	19.00±1.00^{cdef}	19.67±0.58^{abcde}	17.67±0.58^{defghij}
2.	AAUT126	19.67±0.58^{abc}	19.67±1.15^{bcde}	18.33±0.58^{cdefgh}	18.67±1.53^{abcdefg}
3.	AAUT064	19.33±1.15^{abcd}	18.33±0.58^{defg}	18.00±1.00^{defghi}	18.00±1.00^{cdefghi}
4.	AAUT136	20.00±1.00^{abc}	20.00±1.00^{bcd}	17.67±1.15^{efghi}	19.00±1.00^{abcdef}
5.	AAUE132	19.00±1.00^{abcde}	20.00±1.00^{bcd}	20.67±0.58^{ab}	18.00±1.00^{cdefghi}
6.	AAUT134	18.33±1.15^{cdef}	21.00±1.00^{ab}	18.00±1.00^{defghi}	19.00±1.00^{abcdef}
7.	AAUT115	20.00±1.00^{abc}	20.00±1.00^{bcd}	18.33±1.15^{cdefgh}	19.33±1.15^{abcde}
8.	AAUT075	20.33±0.58^{abc}	17.67±1.53^{fghi}	19.00±1.00^{abcdefg}	16.33±1.53^{hijk}
9.	AAUK070	20.00±1.00^{abc}	16.00±1.00^{i}	20.00±1.00^{abcd}	17.33±0.58^{efghij}
10.	AAUT082	19.33±0.58^{abcd}	19.00±1.00^{cdef}	18.33±1.53^{cdefgh}	17.00±1.00^{fghijk}
11.	AAUT081	18.33±1.15^{cdef}	18.33±0.58^{defg}	18.67±1.53^{bcdefgh}	18.00±0.00^{cdefghi}
12.	AAUT101	21.00±1.00^{a}	19.67±1.15^{bcde}	18.67±1.53^{bcdefgh}	20.67±0.58^{a}
13.	AAUT104	18.67±1.53^{bcde}	19.00±1.00^{cdef}	18.67±1.53^{bcdefgh}	16.00±1.00^{ijk}
14.	AAUT121	20.67±0.58^{ab}	18.00±1.00^{efgh}	21.00±1.00^{a}	18.00±1.00^{cdefghi}
15.	AAUE116	17.00±1.00^{efgh}	20.67±0.58^{abc}	18.00±1.00^{defghi}	18.33±0.58^{bcdefgh}
16.	AAUE063	19.00±1.00^{abcde}	17.00±1.00^{ghi}	18.00±1.00^{defghi}	17.67±0.58^{defghij}
17.	AAUE124	20.67±1.15^{ab}	21.00±1.00^{ab}	19.00±1.00^{abcdefg}	18.67±1.53^{abcdefg}
18.	AAUK040	17.33±1.53^{defg}	20.00±1.00^{bcd}	19.67±0.58^{abcde}	17.67±1.53^{defghij}
19.	AAUK135	17.33±1.53^{defg}	18.00±1.00^{efgh}	18.67±0.58^{bcdefg}	18.00±1.00^{cdefghi}
20.	AAUK132	19.00±1.00^{abcde}	20.67±0.58^{abc}	19.33±1.53^{abcdef}	20.33±1.15^{ab}
21.	AAUE144	17.33±1.53^{defg}	19.00±1.00^{cdef}	19.00±1.00^{abcdefg}	19.33±1.15^{abcde}
22.	AAUE051	16.67±2.08^{fgh}	18.00±1.00^{efgh}	19.00±1.00^{abcdefg}	20.00±1.73^{abc}
23.	AAUE048	19.33±1.53^{abcd}	21.33±1.53^{ab}	16.67±1.53^{hij}	16.00±1.00^{ijk}
24.	AAUE142	18.33±1.53^{cdef}	20.67±0.58^{abc}	17.67±1.53^{efghi}	18.33±0.58^{bcdefgh}
25.	AAUK114	21.00±1.00^{a}	22.00±1.00^{a}	20.33±0.58^{abc}	20.67±0.58^{a}
26.	AAUE052	19.00±1.00^{abcde}	17.00±1.00^{ghi}	17.33±0.58^{fghi}	18.67±0.58^{abcdefg}
27.	AAUK122	15.33±0.58^{h}	18.33±0.58^{defg}	17.00±1.00^{ghi}	16.00±1.00^{ijk}
28.	AAUK071	16.33±1.53^{gh}	17.67±1.53^{fghi}	17.67±1.53^{efghi}	16.67±0.58^{ghijk}
29.	AAUK072	19.00±1.00^{abcde}	16.33±1.53^{hi}	17.67±0.58^{efghi}	16.33±1.53^{hijk}
30.	AAUK082	17.33±1.53^{defg}	17.00±1.00^{ghi}	15.00±1.00^{j}	15.00±1.00^{k}
31.	AAUT146	18.33±1.53^{cdef}	17.67±1.53^{fghi}	16.00±1.00^{ij}	15.67±1.53^{jk}
32.	AAUE031	19.67±0.58^{abc}	20.33±0.58^{abc}	20.00±1.00^{abcd}	18.33±0.58^{bcdefgh}
33.	AAUT035	20.67±0.58^{ab}	21.00±1.00^{ab}	19.33±1.15^{abcdef}	20.00±1.00^{abc}
34.	AAUK011	19.67±0.58^{abc}	20.33±1.15^{abc}	20.67±0.58^{ab}	19.67±0.58^{abcd}

$^{a-k}$Means not connected by the same letter within a column are significantly different at $p < 0.05$. Results expressed as average (n = 3) ± SD (standard deviation).

3.4.3.4 Production of Bacteriocins

Among the selected 34 probiotic LAB isolates tested for the production of bacteriocins by agar well diffusion method against some foodborne pathogens, 9 (26.47%) isolates were able to produce bacteriocins (Table 3.8). The average zones of inhibition by which the crude extracts inhibited the growth of the test foodborne pathogens ranged between 11.67 to 17.67 mm. Accordingly, isolate AAUK114 displayed the highest antagonistic activity against *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* (clinical isolate), *Escherichia coli* ATCC 25922 and *Salmonella* Typhimurium with the inhibition zone that ranged from 13.33 to 17.67 mm in diameters. On the contrary, AAUK040 showed a minimum inhibition zone of diameter ranging from 11.67 to 13.33 mm against the test pathogens (Table 3.8).

Explicitly, isolate AAUK040 showed an inhibition zone with a minimum diameter of 12.67 mm, whereas, isolate AAUK114 showed maximum inhibition zone (17.67 mm) against *Listeria monocytogenes* (Table 3.8). Likewise, AAUE048 showed the lowest inhibition zone with a diameter of 13 mm but isolate AAUK114 showed the highest inhibition zone with a diameter of 17.67 mm against *Staphylococcus aureus* ATCC 25923. Isolate AAUK040 and AAUE124 showed 11.67 mm and 15 mm inhibition zone against *Escherichia coli* ATCC 25922, respectively (Table 3.8). Also, 6 isolates showed inhibition zone with a diameter of 12 mm and two isolates showed 13 mm and isolate AAUE124 showed the highest inhibition zone with a diameter of 15.33 mm against *Salmonella* Typhimurium (Table 3.8).

Table 3.8**:** *In vitro* bacteriocin production by potential probiotic LAB isolates

Number	Isolates	Diameter of inhibition zone (mm)			
		L. monocytogenes	***S. aureus***	***E. coli***	***S.* Typhimurium**
1.	AAUE143	14.00±1.00^{de}	16.00±1.00^{abc}	12.33±1.15^{bc}	12.00±1.00^{b}
2.	AAUT064	14.00±1.00^{de}	14.67±1.15^{cd}	12.00±1.00^{c}	12.00±1.00^{b}
3.	AAUT115	15.33±0.58^{cd}	16.00±1.00^{abc}	12.00±1.00^{c}	12.33±0.58^{b}
4.	AAUE124	17.00±1.00^{ab}	17.00±1.00^{ab}	15.00±1.00^{a}	15.33±1.53^{a}
5.	AAUK040	12.67±0.58^{abc}	13.33±1.53^{d}	11.67±0.58^{c}	12.33±1.15^{b}
6.	AAUK135	16.00±1.00^{bc}	15.00±1.00^{bcd}	12.67±1.53^{bc}	12.33±1.53^{b}
7.	AAUK132	16.00±1.00^{bc}	16.67±0.58^{abc}	14.00±1.00^{ab}	13.67±1.53^{ab}
8.	AAUE142	14.00±1.00^{de}	13.00±1.00^{d}	12.00±1.00^{c}	12.00±1.00^{b}
9.	AAUK114	17.67±0.58^{a}	17.67±1.15^{a}	14.67±0.58^{a}	13.33±1.53^{ab}
10.	Control	ND	ND	ND	ND

$^{a-e}$Means with different superscripts within the same column are significantly different ($p<0.05$). Results expressed as average (n = 3) ± SD (standard deviation). ND= Not detected.

The proteinaceous nature of the bacteriocin was confirmed by testing their sensitivity to proteolytic enzymes. The bacteriocin of the 9 screened potential probiotic LAB isolates exhibited complete inactivation of antimicrobial activity after the treatment of bacteriocin with proteinase K, confirming its proteinaceous nature.

3.4.3.5 Antibiotic Susceptibility Testing

A total of 34 candidate probiotic lactic acid bacteria isolates were tested for antibiotic sensitivity or resistance. The results showed that the selected probiotic LAB isolates showed susceptibility to the commonly used antibiotics tetracycline, ampicillin and erythromycin (Table 3.9). On the contrary, all the 34 potential probiotic LAB isolates displayed resistance to kanamycin. Furthermore, 17 (50%) of the potential probiotic lactic acid bacteria isolates showed sensitivity to streptomycin, whereas, the remaining 17 (50%) of the isolates exhibited resistance to streptomycin (Table 3.9).

Table 3.9: Antibiotic susceptibility profile of potential probiotic LAB isolates

Isolates	Diameter of Inhibition Zone				
	Kanamycin	**Streptomycin**	**Tetracycline**	**Ampicillin**	**Erythromycin**
AAUE143	*R*	*S*	*S*	*S*	*S*
AAUT126	*R*	*S*	*S*	*S*	*S*
AAUT064	*R*	*S*	*S*	*S*	*S*
AAUT136	*R*	*S*	*S*	*S*	*S*
AAUE132	*R*	*S*	*S*	*S*	*S*
AAUT134	*R*	*R*	*S*	*S*	*S*
AAUT115	*R*	*S*	*S*	*S*	*S*
AAUT075	*R*	*S*	*S*	*S*	*S*
AAUK070	*R*	*S*	*S*	*S*	*S*
AAUT082	*R*	*S*	*S*	*S*	*S*
AAUT081	*R*	*S*	*S*	*S*	*S*
AAUT101	*R*	*R*	*S*	*S*	*S*
AAUT104	*R*	*R*	*S*	*S*	*S*
AAUT121	*R*	*R*	*S*	*S*	*S*
AAUE116	*R*	*R*	*S*	*S*	*S*
AAUE063	*R*	*R*	*S*	*S*	*S*
AAUE124	*R*	*S*	*S*	*S*	*S*
AAUK040	*R*	*S*	*S*	*S*	*S*
AAUK135	*R*	*S*	*S*	*S*	*S*
AAUK132	*R*	*S*	*S*	*S*	*S*
AAUE144	*R*	*R*	*S*	*S*	*S*
AAUE051	*R*	*R*	*S*	*S*	*S*
AAUE048	*R*	*S*	*S*	*S*	*S*
AAUE142	*R*	*S*	*S*	*S*	*S*
AAUK114	*R*	*S*	*S*	*S*	*S*
AAUE052	*R*	*R*	*S*	*S*	*S*
AAUK122	*R*	*R*	*S*	*S*	*S*
AAUK071	*R*	*R*	*S*	*S*	*S*
AAUK072	*R*	*R*	*S*	*S*	*S*
AAUK082	*R*	*R*	*S*	*S*	*S*
AAUT146	*R*	*R*	*S*	*S*	*S*
AAUE031	*R*	*R*	*S*	*S*	*S*
AAUT035	*R*	*R*	*S*	*S*	*S*
AAUK011	*R*	*R*	*S*	*S*	*S*

The zone of inhibition (diameter in mm) for each antibiotic was measured and expressed as susceptible, S (≥21 mm); intermediate, I (16-20 mm) and resistance R (≤15 mm).

3.4.3.6 Bacterial Adhesion to Stainless Steel Plates

The 34 selected isolates of probiotic LAB were tested for bacterial adhesion to stainless steel plates. The adhesive properties of the potential probiotic LAB isolates were expressed as the percentage of adhering bacterial cells to stainless steel plates (Table 3.10). The adherence ability of the potential probiotic LAB isolates ranged from 29.21 to 41.94% (Table 3.10). Isolate AAUK114 showed the highest (41.94%) adherence rate followed by isolates AAUE124, AAUT115, and AAUK132 with 41.00%, 40.65% and 40.64%, respectively. However, isolate AAUK040 showed the least (29.21%) adherence rate (Table 3.10).

Table 3.10**:** The adhesion property of potential probiotic LAB isolates to stainless steel plate

Number	Source of isolates	Isolates	Survival percent	Survival rate of mean and SD
1.	Ergo	AAUE143	32.59%	32.59 ± 0.98^{ghi}
2.	Teff dough	AAUT126	33.07%	33.07 ± 1.76^{fgh}
3.	Teff dough	AAUT064	35.84%	35.84 ± 0.82^{bcde}
4.	Teff dough	AAUT136	34.10%	34.10 ± 0.77^{cdefg}
5.	Ergo	AAUE132	30.43%	30.43 ± 0.88^{ijk}
6.	Teff dough	AAUT134	29.42%	29.42 ± 1.18^{jk}
7.	Teff dough	AAUT115	40.65%	40.65 ± 1.44^{a}
8.	Teff dough	AAUT075	32.31%	32.31 ± 1.17^{ghi}
9.	Kocho	AAUK070	30.46%	30.46 ± 1.26^{ijk}
10.	Teff dough	AAUT082	33.65%	33.65 ± 1.66^{defg}
11.	Teff dough	AAUT081	30.34%	30.34 ± 1.65^{ijk}
12.	Teff dough	AAUT101	36.73%	36.73 ± 1.53^{b}
13.	Teff dough	AAUT104	35.87%	35.87 ± 1.16^{bcde}
14.	Teff dough	AAUT121	36.59%	36.59 ± 1.58^{bc}
15.	Ergo	AAUE116	30.65%	30.65 ± 1.07^{hijk}
16.	Ergo	AAUE063	32.30%	32.30 ± 0.97^{ghi}
17.	Ergo	AAUE124	41.00%	41.00 ± 1.92^{a}
18.	Kocho	AAUK040	29.21%	29.21 ± 0.92^{k}
19.	Kocho	AAUK135	35.96%	35.96 ± 0.93^{bcde}
20.	Kocho	AAUK132	40.64%	40.64 ± 1.20^{a}
21.	Ergo	AAUE144	35.38%	35.38 ± 2.36^{bcdef}
22.	Ergo	AAUE051	36.11%	36.11 ± 1.24^{bcd}
23.	Ergo	AAUE048	35.32%	35.32 ± 1.28^{bcdef}
24.	Ergo	AAUE142	33.46%	33.46 ± 1.19^{efg}
25.	Kocho	AAUK114	41.94%	41.94 ± 1.64^{a}
26.	Ergo	AAUE052	33.17%	33.17 ± 1.45^{fgh}
27.	Kocho	AAUK122	32.55%	32.55 ± 2.36^{ghi}
28.	Kocho	AAUK071	35.49%	35.49 ± 0.76^{bcdef}
29.	Kocho	AAUK072	34.13%	34.13 ± 1.21^{cdefg}
30.	Kocho	AAUK082	31.73%	31.73 ± 1.13^{ghij}
31.	Teff dough	AAUT146	32.13%	32.13 ± 0.72^{ghi}
32.	Ergo	AAUE031	32.75%	32.75 ± 2.11^{ghi}
33.	Teff dough	AAUT035	33.48%	33.48 ± 1.05^{efg}
34.	Kocho	AAUK011	36.30%	36.30 ± 1.36^{bc}

$^{a-k}$ Means with different superscripts within the same column are significantly different ($p<0.05$). Results expressed as average (n = 3) ± SD (standard deviation).

3.4.4 Molecular Characterization of Probiotics LAB Isolates

The selected 34 potential probiotic LAB isolates were identified as *Lb. plantarum* strains 24 (70.59%), the most abundant strains followed by *Lc. lactis* strains 6 (17.65%) and *Lb. paracasei* strain 3 (8.82%). However, *Lb. brevis* was the least dominant 1 (2.94%) strain. Among the 24 *Lb. plantarum* strains, 13 (54.17%), 7 (29.17%) and 4 (16.67%) strains were isolated from *teff* dough, *kocho* and *ergo*, respectively. All the 6 *Lc. lactis* L105 strains were isolated from *ergo* sample. In addition, from the 3 *Lb. paracasei*, 2 (66.67%) *Lb. paracasei* strains were isolated from *kocho* sample and 1 (33.33%) *Lb. paracasei* was isolated from *ergo* sample. The least dominant strains, *Lb. brevis* was isolated from *kocho* sample.

3.4.4.1 Identification of Probiotic LAB Isolates by 16S rRNA Gene Sequencing

The 16S rRNA gene sequences of the 4 LAB isolates with the best potential probiotic properties showed the highest homology to known species of bacteria in the database (**Error! Reference source not found.**). According to sequencing results, AAUE052 showed a 99% match with *Lb. plantarum* strain JCM 1149, AAUT035 showed 99% homology with *Lb. plantarum* strain CIP 103151, AAUK011 showed 95% similarity with *Lb. paracasei* subsp. *tolerans* strain NBRC 15906 and AAUE031 showed 99% homology with *Lb. paracasei* strain NBRC 15889 (Figure 3).

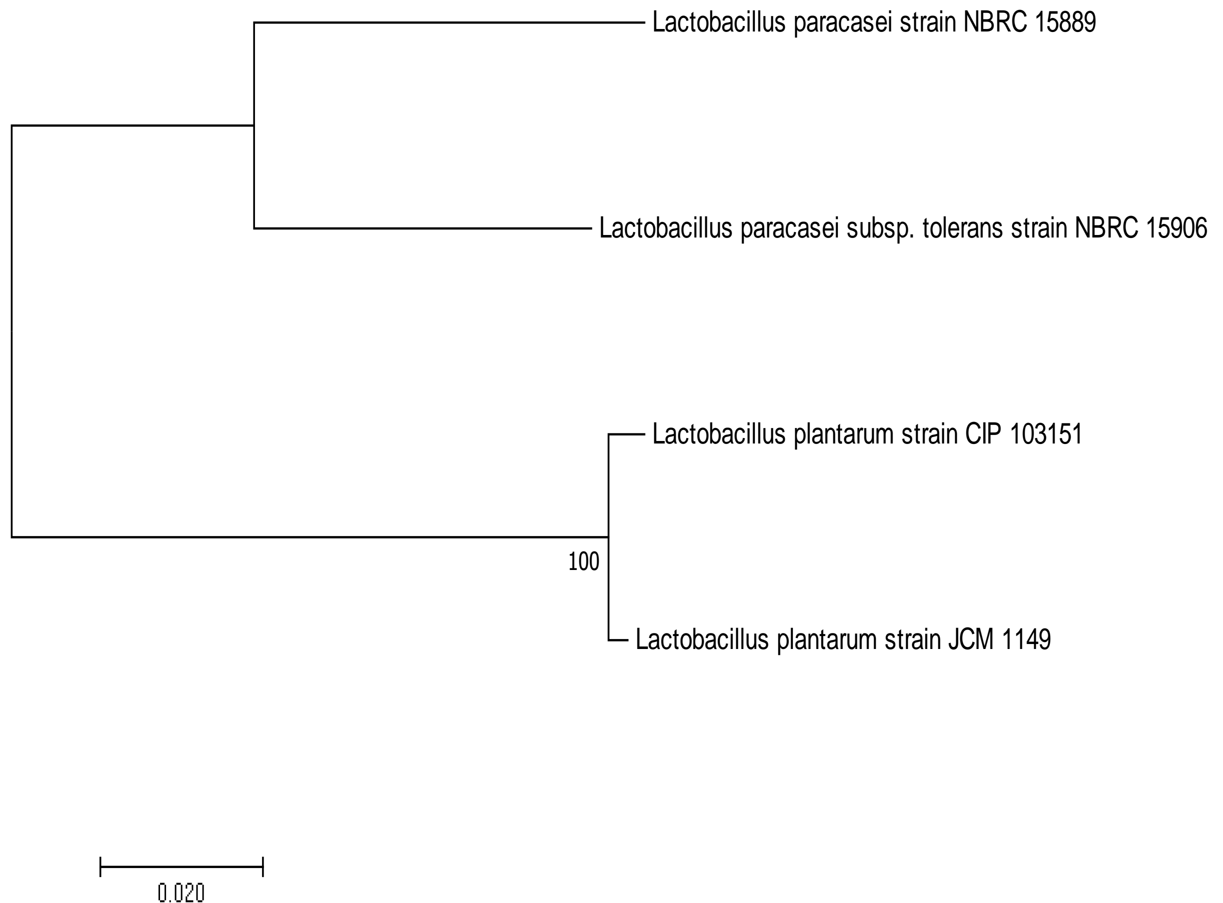

Figure 3.3: Evolutionary relationships of the isolation to the known strains

3.4.4.2 Identification of Probiotic LAB Isolates by Whole Genome Sequencing

The remaining 30 (88.24%) potential probiotic strains were further identified by using whole-genome sequencing to determine genome sizes, genome functioning and species ecology, metabolic potential, influence of geography and strains uniqueness (Table 3.11). Among the strains, 12 (40%) potential probiotic strains were isolated from *teff* dough and 9 (30%) strains from each *ergo* and *kocho*. Accordingly, the 16S rRNA and RecA gene sequences of the 30 selected potential probiotic LAB isolates showed (99 to 100%) homology to known species of bacteria in the database (*Lb. plantarum, Lb. paracasei, Lb.*

brevis and *Lc. lactis*). Hence, out of the total 30 selected potential probiotic LAB isolates, AAUK072 and AAUK114 showed (100%) similarity with *Lb. plantarum* DK0 22T and *Lb. paracasei* R094, respectively (Table 3.11).

The main genomic features determined in this study were reported in Table 3.11. Thus, *Lactobacillus* species had higher G+C content than *Lc. lactis* strains (Table 3.11). Almost all the six genomes of *Lb. lactis* strains isolated from the *ergo* sample had the same G+C content (35.0 to 35.5%). However, the remaining 24 *Lactobacillus* species had higher G+C content (44.2 to 46.1%). Among the strains, *Lb. paracasei* had the highest G+C content (46.1%), followed by *Lb. plantarum* ATCC 14917 (45.9%) and *Lb. brevis* ATCC 14869 (45.8%), which were isolated from *Kocho*, whereas the lowest G+C contents 44.2%, were from *Lb. plantarum* ATCC 14917 that was isolated from *teff* dough (Table 3.11).

Furthermore, the genome sequence of the six *Lc. lactis* strains contained 2,630,489 to 2,717,893 bp and 2,726 to 2,890 candidate coding sequence (CDS; Table 3.11). Similarly, the genome sequence of the 22 *Lb. plantarum* strains contained 2,481,105 to 3,534,554 bp and 2,476 to 4,469 candidate CDS. The genome sequencing of *Lb. paracasei* R094 (AAUK114) and *Lb. brevis* ATCC 14869 (AAUK071) were also displayed 3,134,351 bp and 2,460,308 bp, with 3,280 candidate CDS and 2,547 candidate CDS, respectively. Subsequently, out of the 30 screened potential probiotic strains, the genome sequence of *Lb. plantarum* ATCC 14917 isolated from *teff* dough (AAUT115) contained the highest (3,534,554) bp and candidate CDS (4,469). Whereas, the genome sequence of *Lb. brevis* ATCC 14869 contained the smallest (2,460,308) bp and *Lb.*

plantarum ATCC 14917 (AAUK082) showed the smallest (2,476) candidate CDS (Table 3.11). Generally, the genome sequence of the 30 potential probiotic strains consisted of 45 to 1598 contigs (Table 3.11). Hence, the highest (1,598) contigs was observed in *Lb. plantarum* ATCC 14917 (AAUT115), whereas, the smallest (45) contigs was shown in *Lb. plantarum* ATCC 14917 (K082) (Table 3.11).

Table 3.11: Genomic features of *Lactobacillus* and *Lactococcus* species used in genomic comparisons

Isolates	Species	Strains	Identities	Size (bp)	GC (%)	Contigs	CDS
AAUE143	*Lactococcus lactis*	L105	99.74%	2,669,775	35.5	150	2781
AAUT126	*Lactobacillus plantarum*	ATCC 14917	99.74%	2,962,779	44.7	189	3042
AAUT064	*Lactobacillus plantarum*	ATCC 14917	99.83%	3,190,823	44.4	233	3248
AAUT136	*Lactobacillus plantarum*	ATCC 14917	99.74%	2,990,922	44.6	197	3077
AAUE132	*Lactobacillus plantarum*	ATCC 14917	99.74%	2,819,135	44.8	177	2888
AAUT134	*Lactobacillus plantarum*	ATCC 14917	99.74%	2,962,717	44.7	184	3042
AAUT115	*Lactobacillus plantarum*	ATCC 14917	99.83%	3,534,554	44.2	1598	4469
AAUT075	*Lactobacillus plantarum*	ATCC 14917	99.91%	3,040,971	44.6	201	3103
AAUK070	*Lactobacillus plantarum*	ATCC 14917	99.87%	2,987,637	44.7	182	3054
AAUT082	*Lactobacillus plantarum*	ATCC 14917	99.74%	2,948,780	44.7	199	3033
AAUT081	*Lactobacillus plantarum*	ATCC 14917	99.74%	2,957,867	44.7	226	3062
AAUT101	*Lactobacillus plantarum*	ATCC 14917	99.74%	2,962,770	44.7	181	3033
AAUT104	*Lactobacillus plantarum*	ATCC 14917	99.74%	2,968,304	44.7	185	3052
AAUT121	*Lactobacillus plantarum*	ATCC 14917	99.74%	2,962,504	44.7	179	3031
AAUE116	*Lactococcus lactis*	L105	99.35%	2,680,027	35.0	152	2823
AAUE063	*Lactococcus lactis*	L105	99.35%	2,717,893	35.0	177	2885
AAUE124	*Lactococcus lactis*	L105	99.29%	2,673,196	35.2	176	2863
AAUK040	*Lactobacillus plantarum*	ATCC 14917	99.83%	3,425,501	44.4	113	3428
AAUK135	*Lactobacillus plantarum*	ATCC 14917	99.91%	3,221,109	44.5	56	3199
AAUK132	*Lactobacillus plantarum*	ATCC 14917	99.91%	3,219,555	44.5	46	3193
AAUE144	*Lactococcus lactis*	L105	99.35%	2,630,489	35.5	134	2726
AAUE051	*Lactobacillus plantarum*	ATCC 14917	99.74%	2,819,156	44.8	175	2881
AAUE048	*Lactobacillus plantarum*	ATCC 14917	99.74%	2,961,848	44.7	187	3038
AAUE142	*Lactococcus lactis*	L105	99.35%	2,673,108	35.0	217	2890
AAUK114	*Lactobacillus paracasei*	R094	100.00%	3,134,351	46.1	119	3280
AAUK122	*Lactobacillus plantarum*	ATCC 14917	99.74%	3,089,460	44.5	183	3144
AAUK071	*Lactobacillus brevis*	ATCC 14869	99.54%	2,460,308	45.8	103	2547
AAUK072	*Lactobacillus plantarum*	DK0 22T	100.00%	3,426,101	44.4	115	3429
AAUK082	*Lactobacillus plantarum*	ATCC 14917	99.74%	2,481,105	45.9	45	2476
AAUT146	*Lactobacillus plantarum*	ATCC 14917	99.91%	2,971,733	44.7	281	3100

3.4.4.3 Bacteriocin Gene Cluster Identification

Out of the 30 selected potential probiotic strains, 9 (30%) strains had genes that encode components required for bacteriocin synthesis (Table 3.12). Consequently, two *Lc. lactis* L105 strains (AAUE143 and AAUE142) were predicted to possess a putative class I bacteriocin and *Lc. lactis* L105 (AAUE124) strain exhibited both class I and class II bacteriocins. Similarly, 5 *Lb. plantarum* ATCC 14917 strains shared genes encoding class II bacteriocin proteins. AAUK114 (*Lb. paracasei* R094) was predicted a putative class II and class III bacteriocin protein (Table 3.12). In general, in 9 genomic potential probiotic strains, BAGEL predicted 1 bacteriocin for class III; 3 bacteriocin for class I and 7 bacteriocin for class II (Table 3.12). Moreover, all the selected blast alignments were checked visually to make sure that the alignment is consistent with the presence of a bacteriocin protein.

Table 3.12: Potential probiotic stains sharing different classes of bacteriocin proteins

Isolate code	**Species**	**Strains**	**Bacteriocin**		
			Class I	**Class II**	**Class III**
AAUE143	*Lactococcus lactis*	L105	+	-	-
AAUT064	*Lactobacillus plantarum*	ATCC 14917	-	+	-
AAUT115	*Lactobacillus plantarum*	ATCC 14917	-	+	-
AAUE124	*Lactococcus lactis*	L105	+	+	-
AAUK040	*Lactobacillus plantarum*	ATCC 14917	-	+	-
AAUK135	*Lactobacillus plantarum*	ATCC 14917	-	+	-
AAUK132	*Lactobacillus plantarum*	ATCC 14917	-	+	-
AAUE142	*Lactococcus lactis*	L105	+	-	-
AAUK114	*Lactobacillus paracasei*	R094	-	+	+

Legend: + = positive result, - = negative result.

3.5 Discussion

The study of probiotic properties for application in the preservation of food products and in human health improvement remains to be of great interest. Currently, there is a renewed interest in the use of probiotic LAB due to their antimicrobial properties suggesting them to be likely alternatives to chemical drugs (Rather *et al.*, 2015). Thus, a significant effort has been made to select lactic acid bacteria originating from the traditional Ethiopian fermented food products on the basis of the most important technological, functional and safety criteria in order to obtain potential probiotic LAB.

In the present study, all the selected 34 potential probiotic bacteria were identified as LAB based on their morphological, biochemical and physiological characteristics. Accordingly, some of the probiotic LAB were found to be heterofermentative types, whereas the majority of them were found to be homofermentative types. This finding is in accordance with Asnake Desaleng and Mogessie Ashenafi (2010) who reported that the majority (94%) of the LAB isolated from *awaze*, *qotchqotcha*, and *teff* dough were homofermentative, while a few (6%) of the isolates were heterofermentative. Similarly, Negasi Akalu *et al.* (2017) further reported that the probiotic LAB isolated from traditionally fermented Ethiopian food products (*shamita* and *kocho*) were identified as heterofermentative and homofermentative types.

In this study, the potent LAB showed remarkable tolerance to salt and temperature. Likewise, Azadnia and Khan Nazer (2009) have also reported that LAB isolated from traditional yogurt in tribes of Fars province were able to grow at 4% NaCl concentration but not at 6.5% NaCl concentration. Similarly, Negasi Akalu *et al.* (2017) demonstrated

that all the probiotic LAB isolated from *shamita* and *kocho* were able to grow at 10°C, 15°C but unable to grow at 45°C and most of them were able to grow at 4% and 6.5% NaCl concentration. Kebede Abegaz (2007) also reported that all the strains isolated from Ethiopian fermented *borde* were able to grow at 10°C, 15°C and 37°C incubation, but reduction in growth rate was observed at 45°C.

In the present study, out of the total 260 probiotic LAB isolates tested against pH 2 for 3 and 6 h, only 34 (13.08%) isolates showed tolerance to pH 2 for 6 h. However, the tolerance of the strains was reduced as the incubation time increased to 6 h and the acidic condition lowered to pH 2.0. Thus, the tolerance of the 34 potential probiotic LAB isolates to pH 2.0 for 3 and 6 h showed a survival rate of 33.33 to 96.33%. The candidate probiotic LAB isolates possessed significant variations ($p<0.05$) in their abilities to survive under acidic conditions. Similar to this study, Vinothkanna and Sekar (2019) reported that most of the potential probiotic LAB isolated from fermented polyherbal preparations of Indian Ayurveda showed above 50% survival at pH 2 for 3 h. In line with this, Mourad and Nour-Eddine (2006) have demonstrated that *Lb. plantarum* OL12, *Lb. plantarum* OL9, *Lb. plantarum* OL15 and *Lb. plantarum* OL33 isolated from fermented olives showed survival percentages of 55%, 49%, 65%, and 57%, respectively, when exposed to pH 2.0 for 2 h. Amraii *et al.* (2014) also showed that out of 19 lactobacilli isolated from traditional dairy products and exposed to pH 2 for 2 h, 2 isolates revealed a 40-75% survival rate. However, these results are not in accordance with those reported by previous studies (Negasi Akalu *et al.*, 2017; Rajoka *et al.*, 2017) who indicated that most strains of *Lb. plantarum* isolated from different sources have shown above 80% survival rate at pH 2 for 3 h. Other reports revealed that 5 *Lactobacillus* acid-tolerant strains

shown above 89% survival after exposure to pH 2 for 3 hours (Oh and Jung, 2015). According to Li *et al.* (2019), the survival rates of the five strains isolated from Chinese traditional sourdough incubated for 3 h in simulated gastric juice of pH 2.0, were less than 50%, especially T32 and T17, which completely lost their vitalities.

In the results of present study, out of the 260 LAB isolates from Ethiopian fermented food, only 34 (13.10%) of the isolates were tolerant to pH 2.5 for 3 and 6 h. Similarly, out of 260 LAB isolates, 65 isolates (25.0%) and 41 isolates (15.77%) survived at pH 3 for 3 and 6 h, respectively. Thus, the survival rate of the 34 probiotic LAB isolates at pH 2.5 and 3 for 3 and 6 h was found to different survival rates (64.54-99.32%) and (51.57-96.68%), respectively. Likewise, Negasi Akalu *et al.* (2017) reported that out of 30 LAB isolated from traditionally fermented Ethiopian beverage and food (*shamita* and *kocho*), 17 lactobacilli showed 82 to 97% and 81 to 91% survival rate at pH 2.5 and 3 for 3 and 6 h, respectively. This is also similar to the report of Oh and Jung (2015) who have shown a better survival rate (above 88%) of *Lactobacillus* species in pH 2.5 and 3 for 3 h that were isolated from *omegisool*, a traditionally fermented millet alcoholic beverage of North Korea. As reported by Haghshenas *et al.* (2017), *Lactobacillus* strains isolated from Iranian fermented dairy products survived (71% to 76%) at pH 2.5 for 3 h. From the previous investigations, it has generally been accepted that an isolate with full tolerance to pH 3.0 for 3 h can be considered as high acid-resistant strain with promising probiotic properties (Argyri *et al.*, 2013; Guo *et al.*, 2010). Very recently, Tang *et al.* (2018) reported that all the 9 *Lb. plantarum* strains recovered from the feces of breastfeeding piglets were found to be highly tolerant of pH 3 for 3 h. The acid resistance of the selected isolates in this study was in accordance with the reports of Vinothkanna and

Sekar (2019) who suggested that among the 35 isolates found from fermented *polyherbal* preparations of Indian Ayurveda, 13 showed above 70% survival to pH value of 2.5 for 3 h. A similar phenomenon was found in the research performed by Li *et al.* (2019).

The present results are in contrast to Jermen Mamo *et al.* (2015) who have found low to high survival rates (1.03–100%) for the six *Lactobacillus* species at pH 2.5 and 3.0 for 3 h. The same authors indicated that the maximum survival rate of the six strains was 22.5% at pH 3 for 6 h. Nevertheless, complete loss of viability of lactobacilli isolated from traditional Ethiopian *ergo* was recorded at pH 2.5 for 6 h (Jermen Mamo *et al.*, 2015). Hyronimus *et al.* (2000) have found that moderate survival rates were observed for the four strains with 11–26% after 3 h and 0.2–15% after 6 h. In addition, the current survival rate was higher than that of previously reported strains such as *Lb. plantarum* at pH 3 for 2 and 6 h exposure (Mourad and Nour-Eddine, 2006).

As the results indicate, incubation at low pH resulted in a significant decrease in the survival rate of LAB isolates. These results are in agreement with the finding of Guo *et al.* (2010) who reported that the viable counts of all LAB were significantly affected by low acidity, especially at pH 2. Therefore, when compared to the weak tolerance to low pH seen in some LAB investigated in earlier studies, our isolates had a higher survival rate at pH 2.0, 2.5 and pH 3.0 for a significant period of time. Although acid tolerance in lactobacilli is highly strain-specific, members of the genus *Lactobacillus* are, in general, acidophilic, producing pH 4.0 in foods containing a fermentable carbohydrate (Alakomi *et al.*, 2000).

Acid tolerance is one of the important criteria for the selection of potential probiotic strains. In order to evaluate the beneficial effects of probiotic LAB in the host, they should survive exposure to gastrointestinal (GIT) environment of low pH and other stresses (Begley *et al.*, 2005). The mean transit time of food in the stomach is approximately 3 hours and the stomach acidity varies from person to person naturally and whether an individual has starved prior to ingestion or not (Basturk *et al.*, 2019). Generally, the results of the characterized LAB isolates obtained from traditional fermented foods can be considered as potential candidate in probiotic development since they showed remarkable acid tolerance experienced in human GIT.

LAB isolates were highly resistant to bile salt, which is another indication of their possible survival in the small intestine. All the 34 selected acid-tolerant LAB isolates showed a high tolerance to 0.3% bile salt conditions, ranging from 82.58% to 99.44% survival rate. This is comparable to the bile tolerance of different *Lactobacillus* species isolated from *omegisool*, a traditionally fermented millet alcoholic beverage in Korea (Oh and Jung, 2015). Similar to the present findings, the results in other studies have revealed that all the isolated strains displayed high tolerance to bile salt conditions and the survival rates of *Lactobacillus* strains ranged from 88% to 92% (Haghshenas *et al.*, 2017). In a related study, Negasi Akalu *et al.* (2017) also shown that out of the 30 tested LAB isolates, 17 *Lactobacillus* isolates obtained from Ethiopian traditionally fermented *shamita* and *kocho* showed remarkably high tolerance to an environment containing 0.3% bile salt. Mahasneh *et al.* (2015) reported that most of the *Lactobacillus* species isolated from Jordan traditional fermented products were highly resistant to bile salts at the range of 0.3-2% after 24 h of exposure with little viable count reduction to the level of less than

1 log unit. On the other hand, Boke *et al.* (2010) have indicated that *Lactobacillus* strains B3, G12, A13 and 22 exhibited a low level of tolerance to 0.3% bile salts with a survival rate of 36%, 33 %, 3%, and 3%, respectively.

Generally, bile salts are inhibitory to bacterial cells because they damage the structure of the cell membrane. Therefore, bile salt tolerance is considered as an important selection criterion for probiotic isolates in order to survive the conditions in the small intestine. Furthermore, tolerance to a high bile salt condition is also strain-specific as previously demonstrated (Oh and Jung, 2015). The bile salt tolerance of the strains was expressed as the survival rate of the isolates after incubating for 24 h in MRS broth with bile salt. Furthermore, resistance to bile salt of the isolates could be attributed to their ability to produce bile salt hydrolase (BSH). BSH is an enzyme responsible for deconjugation of bile acids, which are less soluble and less efficiently reabsorbed from the intestinal lumen than their conjugated counterpart. Therefore, deconjugation of bile salts could lead to a decrease in serum cholesterol (Pereira and Gibson, 2002).

Another criterion for functional probiotic is ability to exhibit antagonistic property against pathogens. The selected 34 potential probiotic LAB isolates showed varying degrees of inhibition against *Staphylococcus aureus* ATCC 25923*, Listeria monocytogenes* (clinical isolate), *Escherichia coli* ATCC 25922 *and Salmonella* Typhimurium. According to Handa (2012), isolates having clearance zones ≤9 mm and ≥12 mm diameter against the test pathogens indicated poor and strong antimicrobial activity, respectively.

In accordance with the current study, Bassyouni *et al.* (2012) showed that all of the *Lactobacillus* isolates obtained from Egyptian dairy products had an antibacterial effect against *Escherichia coli* and *Salmonella* Typhimurium where 3 isolates had the most potent antimicrobial activity against the test pathogens with inhibition zone that ranged from 17 to 21 mm in diameters. In agreement with this study, Girum Tadesse *et al.* (2005) demonstrated that all the LAB isolates (n = 118) originated from traditionally fermented Ethiopian beverages (*borde* and *shamita*) belonging to the genera *Lactobacillus, Lactococcus, Leuconostoc,* and *Streptococcus* were found to inhibit the growth of the test strains, such as *Staphylococcus aureus, Salmonella* spp. and *Escherichia coli* O157: H7 with inhibition zone that ranged from 15 to 17 mm in diameters. In line with this, Choi *et al.* (2018) reported that out of the 4 strains of LAB, *Lactobacillus* strain was able to completely inhibit the growth of foodborne pathogens, such as *E. coli* O157: H7 ATCC 35150, *Salmonella enteritidis* KCCM 12021, *Salmonella* Typhimurium KCTC 1925 and *Staphylococcus aureus*.

Ryu and Chang (2013) have revealed that among the 10 potentially probiotic LAB strains isolated from *kimchi*, *Lb plantarum* NO1 inhibited the growth of *Staphylococcus aureus* and *Salmonella* Typhi with inhibition zone that ranged from 13.15 to 16 mm in diameters. The same author showed that all the probiotic LAB strains also inhibited the growth of *Staphylococcus aureus* with inhibition zone ranging from 13.15 to 16 mm in diameters. Negasi Akalu *et al.* (2017) also reported that out of the screened 30 LAB isolated from traditional Ethiopian fermented *ergo,* only 17 isolates belonging to the genera *Lactobacillus* showed antimicrobial activity against some pathogenic bacteria, including *Salmonella* Typhimurium with inhibition zone ranging from 10 to 14.5 mm in

diameters. Haghshenas *et al.* (2017) also reported that among the selected 8 LAB isolated form Iranian fermented dairy products, *Lactobacillus* species, particularly *Lb. plantarum* 15HN, showed the most efficient antagonistic activity and inhibited the growth of *Staphylococcus aureus, Listeria monocytogenes, Salmonella* Typimurium and *Escherichia coli* with inhibition zone diameters of 11.7, 13.7, 12.3 and 12.3 mm, respectively.

Rajoka *et al.* (2017) have verified that all the *Lb rhamnosus* isolated from human milk inhibited the growth of *Staphylococcus aureus, Salmonella* Typhimurium and *Escherichia coli* using agar well diffusion method with variable diameters (6 mm to 14 mm). The results in this study were not in accordance with the report of Mahasneh *et al.* (2015) who revealed that the antagonistic activity of *Lactobacillus* isolates found from local traditional fermented products against *Salmonella* Typimurium and *Escherichia coli* ranged from 26 to 60 mm inhibition zone of diameter.

In the current study, out of the 34 selected potential probiotic lactic acid bacteria, 9 (26.47%) isolates were able to produce bacteriocin into the extracellular culture medium and the activities of bacteriocins produced by the LAB isolates completely disappeared when treated with proteinase K. The experimental results revealed that all the 9 selected isolates were displayed antimicrobial activity of bacteriocin against the foodborne pathogens, where AAUK114 displayed the highest ($p<0.05$) antagonistic activity against *Staphylococcus aureus, Listeria monocytogenes Escherichia coli,* and *Salmonella* Typhimurium with inhibition zone that ranged from 13.33 to 17.67 mm in diameters. The current results are in agreement with Zhang *et al.* (2018) who reported that out of the

total 300 strains isolated from Chinese fermented milk, only six probiotic strains produced bacteriocins and retained antimicrobial activity against *Listeria monocytogenes*. Gaspar *et al.* (2018) have also revealed that the antimicrobial activity of the bacteriocin produced by *Lb acidophilus* KS400 obtained from the filtered culture medium after organic acids and hydrogen peroxide neutralization, showed a clear inhibition zone compared to negative control (no inhibition observed).

Previous studies revealed that bacteriocin-producing *Lactobacillus* species were isolated from various sources including Microbiological Resource Center, Cairo, Egypt (Saad *et al.*, 2015), marine sediments of Parangipettai coast (Elayaraja *et al.*, 2014), and Thai indigenous chickens (Musikasang *et al.*, 2012). Putra *et al.* (2018) have reported that among 57 isolates obtained from Indonesian traditional fermented fish, 27 isolates exhibited inhibition activity and only one isolate (NJ-20) exhibited strong activity against *Staphylococcus aureus* ATCC 6538. However, the neutralized-cell free supernatant of the NJ-20 did not exhibit a clear zone around the well against *Staphylococcus aureus* ATCC 6538. This finding indicates that the neutralized-cell free supernatant did not contain an antimicrobial substance such as bacteriocin.

The antimicrobial activity of probiotics might be caused by the production of antimicrobial compounds such as organic acids, ethanol, carbon dioxide, hydrogen peroxide, short-chain fatty acids, and bacteriocins. Therefore, by producing these antimicrobial compounds, probiotic microorganisms gain an advantage over other microorganisms to survive in the adverse conditions of the gastrointestinal tract (Handa, 2012). In general, these strains harbor the genes needed for the production of

bacteriocins. Hence, the inhibition of the pathogens could be caused by the production of bacteriocins.

All of the tested 34 probiotic LAB strains were found to be resistant to kanamycin but sensitive towards tetracycline, ampicillin and erythromycin. Whereas 17/34 of the isolates were resistant to streptomycin and the other 17/34 of the isolates were sensitive. These results are in agreement with previous antimicrobial resistance pattern amongst *Lactobacillus* species previously isolated from other fermented products (Fitsum Tigu *et al.*, 2016; Rajoka *et al.*, 2017). Similarly, Amraii *et al.* (2014), have indicated that all of the selected LAB strains were sensitive to ampicillin, erythromycin, and tetracycline. Likewise, Ruiz-Moyano *et al.* (2019) have revealed that all the lactobacilli strains isolated from *serpa* cheese, Spain were found susceptible or moderately susceptible to penicillin G, chloramphenicol, erythromycin, tetracycline, ampicillin, gentamycin, and clindamycin. On the contrary, Sukmarini *et al.* (2014) have reported that out of 120 isolates of LAB from four different Indonesian traditional fermented foods, 16 isolates were resistant to erythromycin. In line with this, Pan *et al.* (2011) observed that among the 12 *Lactobacillus* species obtained from Chinese fermented foods, 5 isolates were sensitive to kanamycin; 7 isolates resistant to erythromycin; 9 isolates resistant to ampicillin and 8 isolates were resistant to tetracycline.

Nowadays, antibiotic resistance is considered a serious concern in medicine. According to the European Food Safety Authority (EFSA), intrinsic antibiotic resistance in bacteria presents a minimal risk for horizontal spread whereas acquired resistance is considered high risk (Authority, 2012). In general, although there may be differences between

species and strains, most lactobacilli are generally resistant to antimicrobial inhibitors of nucleic acid synthesis, such as quinolones. However, they are sensitive to cell wall inhibitors and protein synthesis inhibitors, except for vancomycin and aminoglycosides, respectively. It is important to emphasize that resistance to such antimicrobials is intrinsic to the genus *Lactobacillus* and does not present a risk of being transferred through horizontal gene transfer to the bacteria of the native intestinal microbiota. In this context, the observed patterns of antibiotic susceptibility agree with the results obtained in the literature for these *Lactobacillus* species, and they can be used in fermented food (Abriouel *et al.*, 2019).

Among the main important characteristics of probiotic bacteria, adhesion to the intestinal mucosa is required. The current results show that the screened potential probiotic LAB isolates possessed *in vitro* adherence quality to stainless steel plates with the adhesion rate ranging from 29.21 and 41.94%. In accordance with the present study, El-Jeni *et al.* (2015) have reported that the adhesion of probiotic LAB to stainless steel plates ranged from 32 to 35%. Winkelströter *et al.* (2011) have also revealed that pure cultures of *Lb. sakei* ATCC 15521 showed strong adherence to stainless steel surface. Generally, this suggests that our potential probiotic LAB isolates may have a potential capacity to colonize the gastrointestinal (GI) tract mucosa in order to exhibit their remarkable protection effect against enteric pathogens. The nonmetallic materials polypropylene, polystyrene, and glass, covering a wide variety of surface properties (roughness, polar and apolar components), were used as reference. In terms of physicochemical conditions (e.g., pH, ionic strength), the stainless steel has a specific behavior, characterized by

strong adhesion indicating the suitability of the selected LAB isolates for further evaluation of probiotic properties.

The phylogenetic analysis and the 16S rRNA sequencing assigned all the four LAB isolates with probiotic properties to genus *Lactobacillus* and the identified species were *Lb. plantarum* strain JCM 1149, *Lb. paracasei* strain NBRC 15889, *Lb. plantarum* strain CIP 103151 and *Lb. paracasei* subsp. *tolerans* strain NBRC 15906. According to Shokryazdan *et al.* (2014), the results of comparative 16S rRNA gene analysis showed LAB isolates belonged to *Lb. acidophilus, Lb. fermentum, Lb. buchneri,* and *Lb. casei*. In addition, Cho *et al.* (2009) have identified *Lactobacillus* strains with potential probiotic properties from the feces of breastfeeding piglets using 16S rRNA genes analysis. Similarly, 16S rRNA gene sequencing, another recent study by Dowarah *et al.* (2018) have revealed strain-level identification of diverse LAB with probiotic properties isolated from some substrates.

Furthermore, the whole-genome sequencing and assembly confirmed all the 30 isolates with potential probiotic properties to genus *Lactobacillus* and *Lactococcus* and the identified species were 22 *Lb. plantarum* ATCC 14917/DK0 22T strains, 1 *Lb. brevis* ATCC 14869 strain, 1 *Lb. paracasei* R094 strain and 6 *Lc. lactis* L105 strains. Accordingly, the genomic lengths of the *Lactobacillus* and *Lactococcus* species analyzed here are highly variable (from ~3.53 Mb to ~2.46 Mb). In agreement with this study, Stefanovic and McAuliffe (2018) have reported that all the three genomes had a G+C content of 46.3% and a genome size of approximately 3 Mbp, typically observed in *Lb. paracasei*. The same author also revealed that the genome of *Lb. paracasei* DPC2071,

DPC4206 and DPC4536 had 41, 49 and 35 contigs and 2827, 2951 and 2931 number of CDS, respectively. Similarly, Oliveira *et al.* (2017a) have shown that *Lc. garvieae* strains have the highest G+C content, ~38.80%, whereas the lowest G+C contents (~34.86%) was from *Lc*. *lactis* NCDO 2118 and *Lc*. *lactis* KF147, both isolated from vegetables. Additionally, the authors further confirmed that the genome sizes of the *Lactococcus* species ranged from ~1.95 Mb to ~2.60 Mb, and the two *Lc*. *garvieae* strains have the smallest genomes.

Kelleher *et al.* (2017) have reported that the comparison of the 30 lactococcal genomes established an average chromosome length of 2.428 Mbp from a range of 2.250–2.589 Mbp, where it should be noted that the genomes of subsp. *lactis* are larger than their subsp. *cremoris* counterparts. The same investigators indicated that each of the *Lc. lactis* chromosomes generated an overall average of 2344 predicted CDS per chromosome that ranged from 1947 to 2643 CDS. Likewise, Schmid *et al.* (2018) have reported that the draft genome sequence of *Lb. plantarum* strains E2C2 and E2C5 contained 3,603,563 bp and 3,615,168 bp, with GC content 43.99% and 3289 candidate CDS and 43.97% and 3293 candidate CDS, respectively.

In general, the finding that *Lc. lactis* strains have the smallest genomes compared to *Lb. plantarum* strains is in agreement with the lifestyle of *Lc. lactis*, isolated from fermented milk, *ergo*. Because the safety and the source matters for their differences, pathogenic bacteria may scavenge compounds from the host for their own metabolism, they tend to lose genes involved in biosynthetic pathways, thus, presenting smaller genomes (Moran, 2002). However, *Lb. plantarum* strains demonstrating high similarity of G+C content and

genome size as well. The high similarity of the isolates, despite the different sources of isolation, is an indication of their selective adaptation to the gut environment. Additionally, the high similarity at the subspecies level may be related to some specific characteristics already described in literature.

Among the 30 genomic strains used to predict putative bacteriocin, BAGEL4 predicted 1 bacteriocin for class III, 3 bacteriocins for class I and 7 bacteriocins for class II. In accordance with this study, Oliveira *et al.* (2017a) have reported that in *Lc. lactis* NCDO 2118, BAGEL predicted one bacteriocin for each of the three classes. In addition, (Oliveira *et al.*, 2017) indicated that genes that encode components required for bacteriocin synthesis, regulation, and hypothetical proteins were detected in the genome of *Lb. rhamnosus* L156.4. using web-based software BAGEL4, we provided evidence of a putative bacteriocin produced by the 9 probiotic strains isolated from the traditional fermented food products (*teff* dough, *ergo,* and *kocho*), which was further confirmed by *in vitro* assays. The distribution of bacteriocin production in lactococci was previously evaluated and showed that 5% of 280 strains surveyed produced proteinaceous inhibitors (Geis *et al.*, 1983). Bacteriocins are a group of potent antimicrobial peptides produced by some microorganisms including LAB, primarily active against closely related organisms (Parada *et al.*, 2007). This is because the production of bacteriocins depends on the microbial strain and culture conditions. Some LAB are probiotics, others may be potential probiotics or just fermentation cultures that are widely distributed in nature and can be used in the food industry (Ricci *et al.*, 2019). Thus, overall demonstrating all probiotics may not produce bacteriocins and calls for extensive screening scheme in order to isolate more number of strains that are capable of producing the desired bacteriocins.

3.6 Conclusion

In the present study, 24 *Lb. plantarum*, 3 *Lb. paracasei*, 1 *Lb. brevis* and 6 *Lc. lactis* strains from *ergo*, *kocho*, and *teff* dough were found to have promising probiotic properties. It is suggested that these strains can be considered as a good candidate for food industries for prospective development of probiotic cultures with other human health benefits. However, further research work is needed to evaluate the *in vivo* probiotic characteristics of these potential LAB.

Chapter 4 Protective Effect of Potential Probiotic Lactic Acid Bacterial Strains from Some Fermented Ethiopian foods Against *Salmonella* Typhimurium DT104 in Mice

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Abstract

Salmonella is one of the most harmful pathogens responsible for foodborne outbreaks, illnesses and deaths. The aim of this study was to evaluate the effect of potentially probiotic strains against *Salmonella* Typhimurium DT104 in mice. The compatibility test among the selected potential probiotic strains (*Lb. plantarum* AAUK132*, Lb. paracasei* AAUK114 and *Lc. lactis* AAUE124) using the cross-streaking method showed the absence of antagonism. The anti-*Salmonella* activities of co-culture of the isolated potential probiotics in the form of mixed or single culture showed a remarkable anti-*Salmonella* activity with 96.50 to 100% growth inhibition. The combination of strains that showed the highest growth inhibition rates against *Salmonella* Typhimurium DT104, were used to test their effect on the colonization of mice by *Salmonella* Typhimurium DT104. White albino male mice were pre-treated with the mixed potential probiotics for 7 days and infected with *Salmonella* Typhimurium DT104 for 1 day. A total of 3 treatments were applied during which the negative control group was treated with phosphate-buffered saline (PBS) but the positive control group (typ) was challenged with

Salmonella Typhimurium DT104 alone. The treated group (pro-typ) was pre-treated with mixed potential probiotic culture and then infected with *Salmonella* Typhimurium DT104. The survival rate of mice and counts of *Salmonella* in feces were recorded. The survival rate of mice on day 21 after the oral challenge with *Salmonella* Typhimurium DT104 was significantly ($p<0.05$) higher in the experimental pro-typ group (100% survival) compared with the positive control group (20% survival). The counts (colony forming unit per ml) of *Salmonella* in feces were significantly lower ($p< 0.05$) for the pro-typ group compared to the typ group. The combination of potential probiotic strains was able to protect mice against *Salmonella* Typhimurium DT104 infection that demonstrates their potential to be used as probiotic cultures for the production of functional fermented products.

Keywords/Phrases: Probiotic strains, *Salmonella* Typhimurium DT104, *Lactobacillus*

4.1 Introduction

Foodborne diseases (FBD) pose a severe public health problem that significantly affects people's wellbeing and leads to serious socioeconomic implications (Grace, 2016). The major foodborne bacterial pathogens are *Campylobacter jejuni*, *Clostridium perfringens*, *Escherichia coli*, *Listeria monocytogenes, Salmonella* spp. and *Staphylococcus aureus* (Oliver *et al.*, 2009). These pathogens have developed multiple drug resistance and cause great economic losses in developing as well as developed countries (Lengsfeld *et al.*, 2007). The problem of foodborne disease is multifactorial, their prevention and control require multidisciplinary approaches (Sivapalasingam *et al.*, 2004).

Among the major foodborne pathogens*,* *Salmonella enterica* is one of the leading causes of serious illness ranging from acute gastroenteritis to systemic infections including typhoid (Castillo *et al*., 2013). Oral infection with *Salmonella* Typhimurium in mice provokes a disease similar to that caused by *Salmonella* Typhi in humans, with fever, enteritis, and septicemia which is lethal to the host (Santos, 2014). However, the nature and severity of the infection developed depends on many factors, including the serovar involved, the virulence of the strain, the infective dose, the age and immune status of the host. Therefore, it is estimated that *Salmonella* species cause 93.8 million gastroenteritis infections worldwide and resulted in 155 000 deaths each year (Majowicz *et al.*, 2010).

Currently, vaccination and antibiotics are used to prevent and control *Salmonella* infections. Accordingly, antibiotic applications are the common clinical treatments for *Salmonella* infection which, in turn, promotes the development of resistant *Salmonella* species towards antibiotics (Berendonk *et al.*, 2015). In addition, the prolonged use of antibiotics leads to changes in the intestinal commensal microflora (Hammad and Shimamoto, 2010). Due to the occurrence of multidrug-resistant strains and the suboptimal efficacy of currently available vaccines, alternative intervention strategies against *Salmonella* infections are urgently needed (Alcaine *et al.*, 2007; Bhan *et al.*, 2005; Birošova and Mikulašova, 2009). One of the promising alternative control approach is the possible beneficial use of probiotic microbes against various pathogens, including *Salmonella* spp. (Das *et al.*, 2013).

The consumption of a large number of probiotics together with food item can fundamentally promotes the health of the consumers (Hill *et al.*, 2014). The possible

mechanisms by which probiotics protect against enteropathogen infections are the production of antimicrobial substances, competition for limited resources and anti-adhesive effects (Oelschlaeger, 2010). A great number of *in vivo* and *in vitro* studies have been carried out to evaluate the effect of probiotics in the prevention and treatment of gastrointestinal infections caused by *Salmonella* species (Anteneh Tesfaye *et al.*, 2011; Dowarah *et al.*, 2018; GirumTadesse *et al.*, 2005). The beneficial effects of probiotics are known to be genus, species, and strain-specific (Reid *et al.*, 2003; Timmerman *et al.*, 2004). Currently, food-based probiotics are supposed to have supposed greater significance as different food products can harbor native and beneficial probiotics and therefore can be used for both nutritional and therapeutic purposes (Meyer *et al.*, 2007).

Ethiopian fermented food products are well known for their unique fermentation style and can be used as a source of potentially beneficial probiotics. Anteneh Tesfaye *et al.* (2011) revealed the antagonistic effect of LAB strains either as pure or defined mixed-cultures against some foodborne pathogens during fermentation and storage of fermented milk. There are still few research data available on the characterization of probiotic LAB from Ethiopian traditional fermented foods. Most of the traditionally fermented products of Ethiopia are consumed without further heat processing which can be considered as ideal vehicles to carry probiotic bacteria into the human gastrointestinal tract. Probiotic strains isolated from traditionally fermented foods and drinks could have a desirable functional property for their application as probiotics against foodborne pathogens. Thus, the main objective of this study is to test the effect of three potentially probiotic strains of LAB isolated from traditionally Ethiopian fermented *ergo* and *kocho* products against the gut pathogen, *Salmonella* Typhimurium under *in vivo* conditions using laboratory animal.

4.2 Materials and Methods

4.2.1 Bacterial Strains and Growth Condition

The bacterial strains and sources of isolation used in this study are listed in Table 4.13. The potential probiotic strains were isolated from traditionally fermented *kocho* and *ergo* products. These strains were identified as *Lb. plantarum*, *Lb. paracasei* and *Lc lactis* by whole-genome sequencing at Earlham Institute (Norwich, UK) (Unpublished data), non-spore formers and catalase-negative. All the probiotic test strains were able to grow on MRS agar. *Salmonella* Typhimurium DT104 was obtained from Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia (Table 4.13). *Salmonella* Typhimurium was able to grow aerobically in Xylose Lysine Deoxycholate (XLD) agar for 24 h at 37°C.

Table 4.13**:** Source of potential probiotic strains and *Salmonella* Typhimurium DT104 used in this investigation

Number	Designation	Source of isolation	Strains	Accession number
1.	AAUE 124	*Ergo*	*Lactococcus lactis* L105	WBOV00000000
2.	AAUK114	*Kocho*	*Lactobacillus paracasei* R094	WBOW00000000
3.	AAUK132	*Kocho*	*Lactobacillus plantarum* 14917	WBOX00000000
4.	DT104	EPHI	*Salmonella* Typhimurium	

EPHI: Ethiopian Public Health Institute

4.2.2 Compatibility Between Probiotic Strains

The compatibility of the selected potential probiotic strains was examined by a cross-streak method as previously described by Pedersen and Tannock (1989). Overnight

cultures of isolates were streaked perpendicularly by forming across with each other on MRS agar plates. The plates were incubated at 37°C for 48 hours anaerobically using anaerobic jar. The type of growth in the confluence zones (stimulation, inhibition, or absence of interaction between the strains) was visually determined (Sáez *et al.*, 2018). The presence of growth inhibitory halos indicates incompatibility between strains.

4.2.3 Co-culture Assay

To evaluate the effect of selected potential pure strains (*Lc. lactis, Lb. paracasei* and *Lb. plantarum*) and their combinations on the growth of *Salmonella* Typhimurium DT104, liquid co-culture technique was used as described by Potočnjak *et al.* (2017) with some modifications. Before co-cultivation, the selected strains and *Salmonella* Typhimurium were grown separately in MRS broth and Tryptic Soy Broth (TSB), respectively. Thus, 100 µl of each and mixed strains (a total of ca. 10^6 CFU/mL) and of *Salmonella* Typhimurium (10^4 CFU/mL) were inoculated into Brain Heart Infusion (BHI) broth and incubated for 24 h at 37°C. The control was monoculture of *Salmonella* Typhimurium. The number of *Salmonella* Typhimurium was determined by plate count agar on XLD solid medium. Experiments were carried out in triplicates. The inhibition percentage was calculated according to the following equation (Lim and Im, 2009).

$$\text{Inhibition (\%)} = \frac{\text{CFU ml}^{-1}\text{in control} - \text{CFU ml}^{-1}\text{in co incubation culture}}{\text{CFU ml}^{-1}\text{in control}} \text{x100}$$

Therefore, the preparation of pure and mixed probiotic LAB cultures for co-culture assay is presented in Table 4.14.

Table 4.14: Preparation of pure and mixed potential probiotic LAB cultures for co-culture assay

N<u>o</u>	Isolate code	Pure and mixed potential probiotic LAB strains
1.	AAUE124	*Lactococcus lactis*
2.	AAUK132	*Lactobacillus plantarum*
3.	AAUK114	*Lactobacillus paracasei*
4.	AAUE124+AAUK132	*Lac. lactis* + *Lb. plantarum* (mixed 1)
5.	AAUE124+AAUK114	*Lac. lactis* + *Lb. paracasei* (mixed 2)
6.	AAUK132+AAUK114	*Lb. plantarum* + *Lb. paracasei* (mixed 3)
7.	AAUE124+AAUK132+AAUK114	*Lac. lactis* +*Lb. plantarum*+*Lb. paracasei* (mixed 4)

4.2.4 *In Vivo* Antagonistic Effect of Mixed Probiotic Strains Against *Salmonella* Infection

4.2.4.1 Experimental Mice

Four to six weeks old male white albino mice were obtained from the Animal House of Department of Microbial, Cellular and Molecular Biology, Addis Ababa University. Mice were housed in cages in the animal room. These mice were provided with standard diet and water *ad libitum*. The bedding of mice was changed every three days and the health status of the animals was monitored regularly.

4.2.4.2 Experimental Design

A total of 30 male white albino mice were used in the study. Five mice per cage were randomly congregated into three groups. Group I served as a negative control and was treated with phosphate-buffered saline (PBS). Group II was challenged with mono *Salmonella* Typhimurium culture (positive control, typ). Protection against *Salmonella* Typhimurium infection by administration of mixed probiotic cultures was evaluated in

Group III (pro-typ). Fecal samples from each group were pooled and checked for a week for the absence/presence of *Salmonella.* Five gram (g) of fecal material was homogenized in 45 ml of sterile 0.1% buffered peptone water and a total of 25 ml was further enriched in 225 ml of Tryptic Soya broth. Enriched cultures were streaked on XLD plates. After ensuring the absence of *Salmonella* sp. from all groups of mice, *Salmonella* Typhimurium and various LAB cultures were administered to mice after depriving the mice water a day before as indicated by Truusalu *et al.* (2004).

4.2.4.3 Preparation of Treatments

The best performing potential probiotic strains in liquid co-culture assay were selected for *in vivo* test using mouse model. Consequently, *in vivo* evaluation of the probiotic effect of the mixed potential probiotic strains (*Lc. lactis* AAUE124*, Lb. paracasei* AAUK114 and *Lb. plantarum* AAUK132) against *Salmonella* Typhimurium was done via oral gavage of mice. The experiment was conducted twice and the average was used for analysis.

Potential probiotic cultures were separately grown overnight at 37°C in 10 ml of MRS broth. To prepare the mixed culture, the overnight growth of each culture (10 ml) was quantified by serial dilution and plate counting to get population of ca. log 6 $CFUml^{-1}$ that achieved by mixing equal volumes of each strain and was divided into daily portions of combined strains. Prior to the feeding step, the mixed culture was prepared by mixing log 6 $CFUml^{-1}$ of each of the potential probiotic strains. Hence, the mixed potential probiotic strains (*Lc. lactis* AAUE124*, Lb. paracasei* AAUK114 and *Lb. plantarum* AAUK132) were termed as a multi-strain formula (MFA). *Salmonella* Typhimurium was

grown separately overnight at 37°C in 10 ml of Tryptic Soya broth. Overnight growth of *S.* Typhimurium DT104 culture was serially diluted in 10 ml of sterile 0.1% buffered peptone water to give ca. 10^4 CFU ml^{-1}.

The experiment was divided into 3 stages (Setyawardani *et al.*, 2017), initial stage (day 1 to 7), infection stage (day 8) and the final stage (day 9 to 21). Group I mice were given 0.3 ml of phosphate-buffered saline (PBS) for the whole 7 days. Group II were challenged with mono *Salmonella* Typhimurium at day 8 with dose (0.3 ml; 4 log CFU mL^{-1} of viable organism) as one oral dose. Protection (Group III) against *Salmonella* Typhimurium DT104 infection was carried out by the administration of mixed potential probiotic strains. In this Group, mixed LAB strains (0.3 ml per day; 6 log CFU mL^{-1}) were given using a sterile syringe blunt-ended tube for consecutive 7 days. On day 8, the mice were orally administered with *Salmonella* Typhimurium (0.3 ml; 4 logs CFU mL^{-1} of viable organism) as one oral dose. The symptoms and deaths of mice were registered and all the survived animals were killed by cervical dislocation on the 21st day. The percent survival was recorded every day for 21 days.

4.2.5 Viable Cell Counts of *Salmonella Typhimurium* DT104

Total counts of *Salmonella* Typhimurium DT104 were determined by the procedures described by Thushani *et al.* (2003). Aseptically, freshly dropped fecal material of mice was collected daily using sterile forceps from day nine to day 21. Fecal material (5 g) from each Group was moistened for 10 minutes in 45 ml of 0.1% buffered peptone water and then homogenized using a Stomacher lab blender (Stomacher 400, Seward, London, UK). Then appropriate dilutions of each homogenate (0.1 ml) were plated on XLD agar

for enumeration of *Salmonella* Typhimurium DT104. Plates were incubated at 37°C for 24 hours and colony-forming units on the plates were recorded. When counts were < log 1 CFU mL^{-1}, samples were enriched in Tryptic Soya broth. Each determination was done in triplicate.

4.3 Data Analysis

All experiments were carried out in triplicate. The results were expressed as mean standard deviation (SD). Statistical analysis was performed using SAS software R 9.1 (SAS Institute Japan, Tokyo) and Stat View Ver. 5 (SAS Institute, Cary, NC).

4.4 Results

4.4.1 Compatibility among Probiotic Strains

Compatibility among 3 selected potential probiotic strains was determined by cross-streaking the probiotic strains *(Lb. plantarum* AAUK132*, Lb. paracasei* AAUK114 and *Lc. lactis* AAUE124) on MRS agar plate (Table 4.15). Although the selected potential probiotic strains were confirmed to have antagonistic activity towards the test pathogen, the 3 selected potential probiotic strains did not show any inhibition halos against each other (Table 4.15). Finally, after the compatibility experiment, the 3 potential probiotic strains isolated from traditional fermented food products were taken to the next step for the co-culture assay study.

Table 4.15**:** The interaction between the selected potential probiotic LAB strains

No	Isolate code	Mixed probiotic strains	Inhibition	Co-growth
1.	AAUE124-AAUK132	*Lc. lactis-Lb. plantarum*	-	+
2.	AAUE124-AAUK114	*Lc. lactis-Lb. paracasei*	-	+
3.	AAUK132-AAUK114	*Lb. plantarum-Lb. paracasei*	-	+
4.	AAUE124-AAUK132-AAUK114	*Lc. lactis-Lb. plantarum-Lb. paracasei*	-	+

Legend: - = negative for growth, + = positive for growth.

4.4.2 Co-culture Assay

All the pure and mixed potential probiotic strains when separately and/or in combined forms co-cultured against the test foodborne pathogen (*Salmonella* Typhimurium DT104), there was more than 96% growth inhibition of the test organism (Table 4.16).

The highest co-culture antagonistic activity (100% growth inhibition) was observed with the mixed cultures of the three probiotic strains (mix 4) followed by the combination of two combined probiotic strains in the form of mix 2, mix 3 and mix 1 with 99.74%, 99.72% and 99.71% of inhibition, respectively. However, the lowest (96.50%; $p<0.05$) growth inhibition was shown with the separate pure culture of *Lb. plantarum* against *Salmonella* Typhimurium DT104 (Table 4.16).

Table 4.16**:** Co-culture assay of potential probiotic LAB strains against *Salmonella* Typhimarium DT104

No	**Isolate code**	**Probiotic strains**	**Inhibition (%)**
1.	AAUE124	*Lactococcus lactis*	97.11 ± 0.23^{b}
2.	AAUK132	*Lactobacillus plantarum*	96.50 ± 0.29^{c}
3.	AAUK114	*Lactobacillus paracasei*	97.27 ± 0.26^{b}
4.	AAUE124+AAUK132	*Lac. lactis+Lb. plantarum* (mix 1)	99.71 ± 0.02^{a}
5.	AAUE124+AAUK114	*Lac. lactis+Lb. paracasei* (mix 2)	99.74 ± 0.02^{a}
6.	AAUK132+AAUK114	*Lb. plantarum+Lb. paracasei* (mix 3)	99.72 ± 0.01^{a}
7.	AAUE124+AAUK132+AAUK114	*Lac. lactis+Lb. plantarum+Lb. paracasei* (mix 4)	100.00 ± 0.00^{a}

Data are means ± SD from three replications and values followed by different letter within the same columns indicates a significant difference ($p< 0.05$).

4.4.3 *In Vivo* Antagonistic Effect of Mixed Probiotic Strains Against *Salmonella* Infection

The survival rate of the treated Group (pro-typ) was 100%, whereas only 20% of the positive control group that was challenged only with *Salmonella* Typhimurium DT104 survived (Figure 4.4).

Apparently, day 1 post-infection with *Salmonella* Typhimurium DT104, all the mice in Group pro-typ and positive control Group (typ) started to show disease symptoms. Ultimately, hair erection and diarrhea were observed. However, the mice pretreated with the mixed potential probiotic strains were able to recover from sickness, but the mice challenged with *Salmonella* Typhimurium DT104 alone (positive control group) became sick and finally died. Therefore, in the positive control group (Group II), the first mortality rate (30%) was observed at day 3 and at day 5 by increasing the death rate to 50% (Figure 4.4). As testing time extended beyond five days, the mortality rate was increasing in the positive control. Finally, the mortality rate (80%) in mice was recorded at the end of day 21. However, there was no mortality in the negative control group I only treated with the PBS (Figure 4.4).

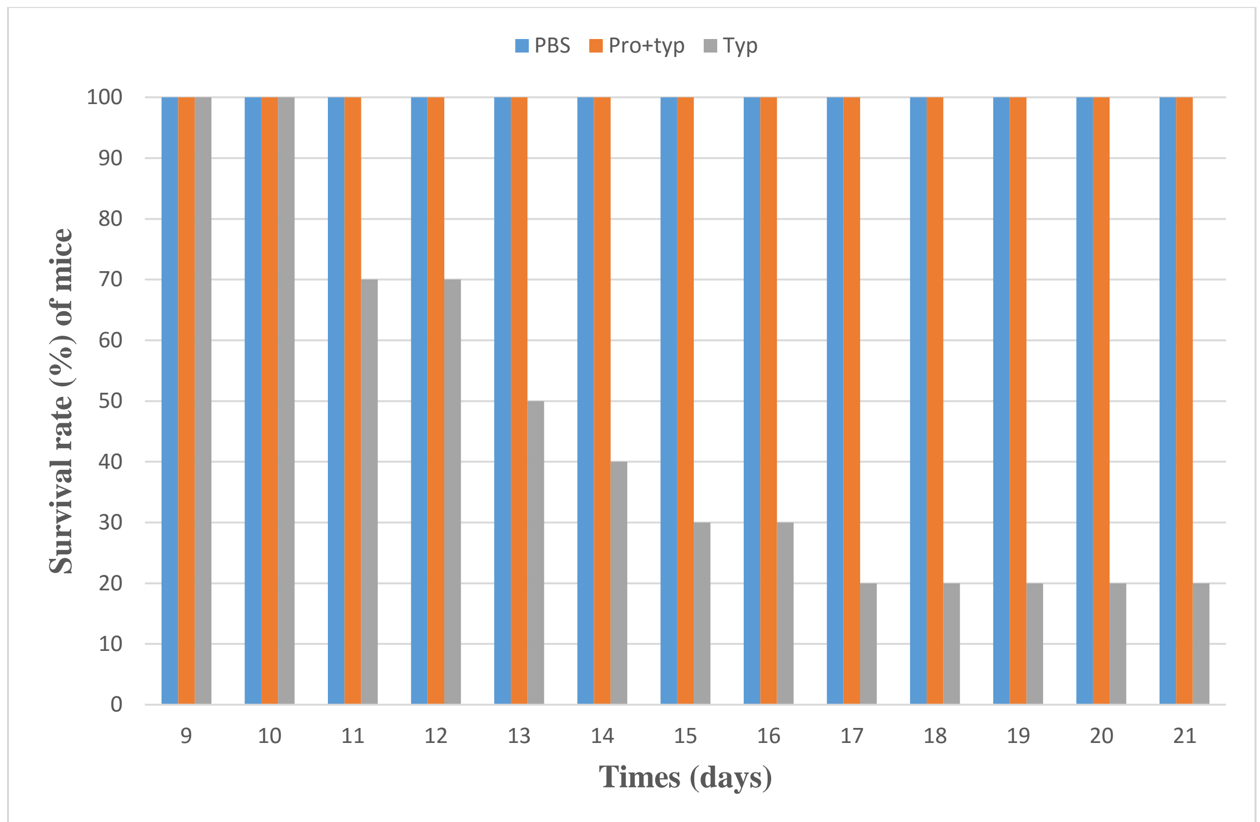

Figure 4.4**:** Survival of mice treated or not with mixed potential probiotic strains and orally infected with *Salmonella* Typhimurium DT104. Results expressed as average (n = 2) ± SD (standard deviation).

4.4.4 Viable Cell Count of *Salmonella Typhimurium* DT104 in Fecal Material

In the negative control group (PBS) of mice, no viable *Salmonella* Typhimurium DT104 counts were detected in the fecal samples of the mice (Table 4.17). In comparison with the results from Group II, *Salmonella*-challenged mice that fed on mixed probiotic strains for 7 days reduced ($p<0.05$) the *Salmonella* cells in the feces when measured from day 9 to day 21 (Table 4.17). The findings showed that the CFU counts of *Salmonella* Typhimurium DT104 in mice which were given with mixed probiotics strains were lower than that of the positive control group. On the other hand, in the feces of mice fed with

combined probiotic LAB strains, the counts of *Salmonella* Typhimurium DT104 cells was significantly reduced from 2.30 to 0.00 log CFU ml^{-1}. However, the noticeable effect of treatment with the combined potential probiotic LAB strains was observed from day 18 and onwards (Table 4.17).

Generally, the counts of *Salmonella* Typhimurium DT104 from the feces of the positive control Group (typ) were consistently higher throughout the experiment of post-infection than from the feces of probiotic-treated mice group (Table 4.17). Hence, when the selected potential probiotic LAB strains were administered at a dose of log 6 $CFUml^{-1}$ level, *Salmonella Typhimurium* DT104 was eliminated from the feces of probiotic-treated mice Group at day 20 and onwards (Table 4.17). Thus, in comparison with the results from the Group I, there was no difference observed from days 20 and 21 in the mice treated with mixed probiotic strains since there was no viable cell count of the test pathogen (Table 4.17). But the recovery rate of viable cell counts in feces of mice challenged only with *Salmonella* at day 20 was observed to be 4.53 log CFU ml^{-1}.

Table 4.17**:** Viable cell counts of *Salmonella* Typhimurium in fecal material of mice

Sample	**Sampling days**	**Treatment group**		
		Negative control (PBS)	**Pro-typ**	**Positive control (typ)**
Fecal material of mice	Day 9	0.00 ± 0.00^{a}	2.27 ± 0.01^{ab}	4.47 ± 0.02^{f}
	Day 10	0.00 ± 0.00^{a}	2.30 ± 0.03^{a}	4.52 ± 0.04^{e}
	Day 11	0.00 ± 0.00^{a}	1.93 ± 0.04^{abc}	4.56 ± 0.01^{bcd}
	Day 12	0.00 ± 0.00^{a}	1.90 ± 0.07^{abc}	4.55 ± 0.02^{cde}
	Day 13	0.00 ± 0.00^{a}	1.88 ± 0.04^{abc}	4.58 ± 0.02^{abc}
	Day 14	0.00 ± 0.00^{a}	1.82 ± 0.05^{bcd}	4.61 ± 0.00^{a}
	Day 15	0.00 ± 0.00^{a}	1.74 ± 0.06^{cd}	4.60 ± 0.01^{a}
	Day 16	0.00 ± 0.00^{a}	1.65 ± 0.07^{cd}	4.59 ± 0.00^{ab}
	Day 17	0.00 ± 0.00^{a}	1.65 ± 0.07^{cd}	4.56 ± 0.01^{bcd}
	Day 18	0.00 ± 0.00^{a}	1.39 ± 0.13^{d}	4.56 ± 0.01^{bcd}
	Day 19	0.00 ± 0.00^{a}	0.50 ± 0.71^{e}	4.54 ± 0.01^{de}
	Day 20	0.00 ± 0.00^{a}	0.00 ± 0.00^{f}	4.53 ± 0.01^{de}
	Day 21	0.00 ± 0.00^{a}	0.00 ± 0.00^{f}	4.52 ± 0.02^{e}

Data are means ± SD from three replications and values followed by a different letter(s) within the column indicate significant differences ($p < 0.05$).

4.5 Discussion

The cross-streak plate method showed that the selected potential probiotic strains were found compatible. While assessing potential multi-strain probiotic cultures, it is essential to carry out compatibility tests in order to avoid the combining of strains showing antagonistic effects against each other. The present results are in agreement with those obtained by Sáez *et al.* (2018), who reported the absence of inhibition halos in selected LAB cell-free supernatants against each other when combined in a mixed culture form. Likewise, Farouk Mohamed *et al.* (2017) have revealed that the 5 selected probiotic strains did not show any inhibitory effect on each other under *in vivo* conditions.

Probiotics have been successfully used for the prevention and treatment of various gastrointestinal diseases of humans and animals (Fayol-Messaoudi *et al.*, 2005). The beneficial effect of probiotic strains present in the fermented food products was recognized to have a nutritional and therapeutic effect on human health (Meyer *et al.*, 2007). Several *in vivo* and *in vitro* studies have demonstrated that probiotics can inhibit *Salmonella* associated diarrhea (Adetoye *et al.*, 2018; Anteneh Tesfaye *et al.*, 2011; Dowarah *et al.*, 2018; Girum Tadesse *et al.*, 2005). In the present study, the relevant functional characteristics of these potential probiotic strains (*Lc. lactis* AAUE124, *Lb. paracasei* AAUK114 and *Lb. plantarum* AAUK132 and their combinations) showed effective inhibitory activities against *Salmonella* Typhimurium DT104 mainly in co-culture experiments.

All three potential probiotic strains and their combinations were able to remarkably inhibit the growth of *Salmonella* Typhimurium DT104 under *in vitro* conditions of co-

culturing assay. The highest inhibition (100%; $p<0.05$) was observed with the combination of the three probiotic strains as in the form of mix 4 (*Lc. lactis* AAUE124, *Lb. paracasei* AAUK114 and *Lb. plantarum* AAUK132). In agreement with this study, Adetoye *et al.* (2018) have revealed that *Lb. salivarius* C86 and *Lb. amylovorus* C94 strains obtained from cattle feces were able to inhibit the growth of *Salmonella* spp. completely between 8 and 16 hours of co-incubation with no recoverable *Salmonella* spp. in the growth medium. Different authors have also reported a strong inhibition of *Salmonella* spp. by LAB in co-culture assay (Abdel-Daim *et al.*, 2013; Coman *et al.*, 2014; Dowarah *et al.*, 2018). In addition, Potočnjak *et al.* (2017) have reported that all the tested *Lb. plantarum strains (*A, B and S1) were able to inhibit ($p< 0.05$) the growth of *Salmonella* Typhimurium at all-time points (6, 12 and 24 h) in co-culture assay. The same author reported that after 24 h of co-cultivation, the number of *Salmonella* cells was reduced 1000 times in comparison with *Salmonella* monoculture and the inhibition was most pronounced after 12 h of co-incubation and amounted to 97, 98 and 94% by strains A, B, and S1, respectively.

In the present study, oral administration of lactic acid bacteria to model mice caused complete inhibition of the test pathogen, particularly when used in combined forms. Oral administration of potential probiotic LAB strains has a beneficial effect on maintaining and improving host health (Liu *et al.*, 2018). Viable probiotic strains are used in most probiotic studies, while few studies using heat-killed probiotic bacteria have been reported (Ishikawa *et al.*, 2010). Several studies (Dowarah *et al.*, 2018; Júnior *et al.*, 2018; Liu *et al.*, 2018) indicated that probiotic LAB have protective effects against *Salmonella* infections by involving a number of possible mechanisms. Consequently, the

protective mechanisms of inhibition by viable probiotic LAB encompass antimicrobial compounds produced by probiotic bacteria that can kill enteric pathogens directly in the gastrointestinal tract (Aiba *et al.*, 1998); enhanced host intestinal immunity by increasing secretory IgA production to eliminate enteric pathogens (Tejada-Simon *et al.*, 1999) and competitive inhibition by binding to receptors used by pathogens on epithelial cells such as mannose and glycoproteins (Chiu *et al.*, 2008).

The mortality rate of the challenged model mice with *Salmonella* Typhimurium DT104 was prevented by pretreatment with mixed probiotic LAB strains (*Lc. lactis* AAUE124*,* *Lb. paracasei* AAUK114 and *Lb. plantarum* AAUK132). In agreement to the current study, Moura *et al.* (2001) reported that the survival rate of mice supplemented with *Lb. acidophilus* UFV-H2B20 and then infected with *Salmonella Typhimurium* was significantly ($p<0.05$) higher in the experimental group (34.6% survival) compared to the control group (0% survival). In a recent study (Liu *et al.*, 2018), the potential of anti-*Salmonella* activity, mice were assessed using model mice that were orally supplemented with a mixture of *Lb. plantarum* for 10 days followed by infection with *Salmonella* Typhimurium SL1344. As a result, the survival rate of the *Lb. plantarum*-pretreated group was 60% at 15 days post-infection, whereas that of the infected group was only 40%. Moreover, Júnior *et al.* (2018) have revealed that higher survival rate (70%) was observed in mice that were promptly treated with oral administration of *Lb. diolivorans* 1Z and challenged with *Salmonella* Typhimurium in comparison with mice receiving only water and then challenged with *Salmonella* Typhimurium where the latter group showed 0% survival rate.

In the present study, the mixed probiotic cultures eliminated the test pathogen from the feces of probiotic-treated mice groups when administrated at log 6 CFU/ml inoculum level at day 20. However, the elimination of *Salmonella* Typhimurium DT104 took quite a long time in the experimental mice. This could be due to the time required for a probiotic strain to colonize the intestine in order to play their probiotic roles. On the contrary, mice without probiotics (typ) showed a high population of *Salmonella* Typhimurium DT104 on day 20. In general, regular consumption of fermented products containing probiotic cultures would result in the establishment of these cultures in the human intestine, which may help the rapid elimination of an enteric pathogen in the intestine. Furthermore, the reduction of intestinal *Salmonella* numbers due to the effects of probiotics was reported by different workers (Collins *et al.*, 2010; Eom *et al.*, 2015; Ishikawa *et al.*, 2010). Evidently, *Salmonella* Typhimurium ATCC 14028 was completely excluded within 23 days in mice when *Lb. rhamnosus* and *Lb. plantarum* were used as probiotic (Setyawardani *et al.*, 2017). Similarly, oral administration of a combination of selected lactic acid bacterial strains had a significant protective effect on *Salmonella* invasion and inflammation in broiler chicks (Chen *et al.*, 2012).

Correspondingly, the mixed strains of *Lb plantarum* had preventive effects against *Salmonella* infection as they decreased *Salmonella*-induced animal deaths in a mouse model (Liu *et al.*, 2018). In earlier study administering a five-strain probiotic combination as either a milk fermenter or milk suspension for a total of 30 days significantly reduced *S.* Typhimurium infection in probiotic-treated pigs at 15 days post-infection (Casey *et al.*, 2007). Moreover, the protective effect of *Lb. rhamnosus* GG

against *Salmonella* infection in mice was significantly different at 4, 7, and 11 days post-inoculation with *Salmonella* Typhimurium C5 (Hudault *et al.*, 1997).

4.6 Conclusion

Ethiopian fermented food products are rich in potential probiotic LAB strains that may exhibit antimicrobial efficacy against foodborne pathogens due to their bactericidal properties. In the present study, the selected potential probiotic LAB strains (*Lc. lactis* AAUE124, *Lb. paracasei* AAUK114 and *Lb. plantarum* AAUK132) were able to show a protective effect against *Salmonella* Typhimurium DT104 infection in experimental mice. As the findings suggest, their promising potential probiotics could be used for the production of functional fermented products.

Chapter 5 Bacterial Diversity of Some Traditionally Fermented Ethiopian Food Products

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Abstract

Fermented foods play a major role in the diet of people, where a wide variety of raw materials that are fermented in Ethiopia. To study the microbial diversity of these traditional Ethiopian fermented food such as *teff* dough, *ergo* and *kocho* products, culture-independent method, particularly 16S rRNA amplicon sequencing were applied. The sequencing of the amplicons resulted in an average of 76,594.33 ± 2,876.25 high-quality reads per sample. The bacterial communities of the three traditional fermented foods were dominated by the phylum Firmicutes, followed by Proteobacteria and Actinobacteria. At the genus level, the prevalent microorganisms were *Lactobacillus*, *Zymomonas, Streptococcus*, *Leuconostoc*, *Bacillus* and *Weissella. Lactobacillus* was the most abundant genus in fermented *teff* dough and *kocho* products, whereas *Zymomonas* the most abundant genus in fermented *ergo* followed by *Lactobacillus* genus. In general, these results indicate a high level of diversity in the bacterial community structure in

traditional fermented foods than previously known and demonstrate the possible influences on food preparation processes, and/or the type of raw materials used on bacterial diversity.

Keywords/Phrases: Fermented foods, DNA extraction, 16S rRNA gene metagenomics

5.1 Introduction

Traditional fermented foods play a major role in the diet of numerous societies in the world, and Africa is perhaps the continent with the richest varieties of fermented foods. Fermentation is known to play a major role in combating food spoilage and foodborne diseases (Tamang and Kailasapathy, 2010). In fact, traditional fermentation is still a largely home-based process used throughout the continent. A wide variety of raw materials are traditionally fermented in different countries of Africa, which have been classified into groups that include fermented non-alcoholic cereals, starchy root crops, animal proteins, vegetable proteins and alcoholic beverages (Franz *et al.*, 2014; Tamang and Kailasapathy, 2010). Likewise, in Ethiopia, fermented food and beverage products form an important part of the diet. Hence, these fermented food products are made from plant and animal materials in which bacteria, yeasts and molds play a significant role by modifying the material physically and/or nutritionally.

Food associated microorganisms are widely distributed in the environment. These microbes play a central role in the regulation of fermentation processes and have an important effect on people's lives. However, it is estimated that 99% of microorganisms observable in nature typically are not cultivated using standard techniques (Amann *et al.*,

1995). For a large number of microbes that are unknown and unculturable, a culture-dependent method prejudices the real understanding of microbial diversity in nature. Hence, culture-independent metagenomic methods, particularly amplicon sequencing, are increasingly used to comprehend the microbial ecology of naturally fermented food products. The information obtained from culture-independent methods can be used to identify biomarkers to assess the quality of fermented foods (Walsh *et al.*, 2017). A great number of metagenomic analyses have been carried out to evaluate the bacterial composition in traditional fermented food products (Addisu Fekadu *et al.*, 2018; Liu and Tong, 2017; Tesfemariam Berhe *et al.*, 2019). In recognition of the significance of microorganisms in the production of quality fermented foods, extensive studies have been carried out in order to use these microbes in the development of new functional foods (Liu and Tong, 2017). A number of genera and species of microbes in different fermented foods such as fermented milk, vegetables, cereals, legumes, meats and fish have been isolated mainly using culture-dependent methods (Tamang *et al.*, 2016). Although this approach may not give the real diversity of microflora associated with foods and there is a need for a holistic approach.

Culture-independent methods can provide valuable information about fermentation dynamics in fermented products. Therefore, some studies on bacterial community composition in traditionally Ethiopian fermented food products were undertaken by few workers (Addisu Fekadu *et al.*, 2018; Tesfemariam Berhe *et al.*, 2019). However, detailed investigations of bacterial community variations in different Ethiopian fermented foods remain scarce. It is, therefore, necessary to acquire a better and more accurate understanding of the microbial community in traditional fermented foods derived from

various regions in the country. The study of the microbial community is also relevant in biotechnology as it is the basis for not only the improvement of fermentation processes but also for the understanding of the microbial interactions that drive the best quality process. The availability of such a powerful toolbox offers good opportunities to study food microbes by understanding how their potential functions can be changed or modulated with the ultimate goal of improving food quality. Hence, the major purpose of this study was to assess the diversity of the native bacterial communities among the 3 conventional fermented foods using 16S rRNA sequencing. The results would improve our understanding of the differences in bacterial diversity among the samples so as to select optimal and appropriate functional bacteria for the manufacturing of high-quality and distinctive fermented food products.

5.2 Materials and Methods

5.2.1 Sample Collection

Three samples of traditionally fermented food products were aseptically collected from Addis Ababa and its surroundings by using sterilized bottles. The samples were obtained from small scale producers. The samples were brought to the laboratory with an icebox and stored at -20°C until the DNA was extracted.

5.2.2 Pre-processing, DNA Extraction, and DNA Quantification

DNA extraction was performed using the FastDNA SPIN Kit for soil with a pre-processing treatment, which separates the microbial cells from large solid particles present in the sample. First, 20 g of the food sample was separately mixed with 10 ml of

cold ultrapure H_2O by vigorous vortexing. The solid particles were removed by centrifugation at 800 x *g* for 1 min at 4°C and the supernatant was retained. A further 10 ml H_2O was added and the process was repeated three times in total and a final volume of approximately 30 ml supernatant was obtained. Cells were harvested from the particle-free supernatants by centrifugation at 3000 x *g* for 20 min at 4°C. The supernatant was discarded and the pellet was washed three times using 1 ml PBS buffer. After centrifugation at 14,000 x *g* for 2 min, the pellet was re-suspended in 978 µl sodium phosphate buffer and 122 µl MT, both provided in the kit, incubated for 1 h at 4°C and homogenized for 60 s at a speed setting of 6.5 m/s, using a FastPrep-24 instrument (MP Biomedicals, UK). This process was repeated three times and the samples were kept on ice for 5 min between each homogenization step. DNA extraction was performed according to the manufacturer's instructions. Samples were re-suspended in 50 µl elution buffer. Total DNA extracted from the fermented samples was quantified fluorometrically by a Qubit 3.0 fluorometer (Invitrogen, Carlsbad, CA) using the Qubit dsDNA BR Assay Kit (Invitrogen), or the Qubit dsDNA HS Assay Kit (Invitrogen) when the concentration of DNA was <10 ng/µl.

3.2.3 DNA Electrophoresis

A 1% agarose gel was prepared in 0.5 TBE and boiled in microwave to melt agarose. The agarose was cooled until hand-heat (55-60°C) and poured into a gel former with comb. After solidification, 1 µl 10x gel loading buffer was added to the sample (10 µl). The gel was stained for 30 mins in 1 µl/ml ethidium bromide and rinsed in water. Finally, the gel was viewed under UV illumination.

5.2.4 Illumina High-Throughput Sequencing

A 16S rRNA genes PCR amplification and sequencing were performed by Novogene (Hong Kong). The V4 hypervariable region of the 16S rRNA gene was amplified using specific primers 515F and 806R (Caporaso *et al.*, 2011). All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs, USA). The libraries generated with TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, USA) were sequenced using paired-end Illumina sequencing (2 × 250 bp) on the HiSeq2500 platform (Illumina, USA).

5.2.5 Sequence Analysis

Sequencing data were analyzed using the Quantitative Insights into Microbial Ecology 2 (QIIME2) 2018.8 software (Caporaso *et al.*, 2010). The demultiplexed paired-end reads were filtered of substitution and chimera errors and merged using DADA2 (Callahan *et al.*, 2016). Bacterial taxonomic assignment was performed at 97% similarity index using a Naive Bayes classifier trained on the Silva version 132 99% operational taxonomic units (OTUs) database (Quast *et al.*, 2012), where the sequences were trimmed to only include 250 bases from the V4 region bound by the 515F/806R primer pair.

5.3 Data Analysis

The results are presented as the mean ± standard deviation (SD). Sequencing data were analyzed using the Quantitative Insights into Microbial Ecology 2 (QIIME2) 2018.8 software.

5.4 Results

5.4.1 Bacterial Diversity

The sequencing resulted in an average of 89,816.33 ± 3,629.62 sequences per sample (Table 5.18). Of these, 14.72% were discarded due to quality issues, reads not merging, or after being identified as chimeras; as a result, high-quality sequences were analyzed with an average of 76,594.33 ± 2,876.25 sequences per sample. Any background DNA was removed by filtering sequences assigned to chloroplast and mitochondrial taxonomic groups. Data were rarefied to 75,890.67 ± 3,385.26 sequences per sample to avoid bias (Table 5.18).

Table 5.18**:** Number of sequences analysed for all the studied traditional fermented food samples

Sample code	Sequence count	filtered	Denoised	merged	non-chimeric	non-background DNA
S34	89739	85444	85444	82499	77529	77402
S35	93484	87636	87636	85001	78887	78257
S36	86226	82264	82264	79919	73367	72013

The sequences obtained from the three traditional fermented food samples correspond to one domain, bacteria (Table 5.19). The microbial communities in the three traditional fermented foods were composed of different prokaryotic cells with organisms belonging to different phyla. Accordingly, a total of 12 phyla were observed in the three studied food samples (Table 5.19). The 11 different phyla were observed in samples S34 and S36

and 12 phyla in sample S35. Among them, 10 phyla were in common in the three studied samples, which corresponded to: Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Cyanobacteria, Fusobacteria, Tenericutes, Spirochaetes, Saccharibacteria and Deferribacteres (Table 5.19). However, the phyla Synergistetes and Chloroflexi were found only in sample S34 and S36, respectively. Furthermore, phyla Verrucomicrobia and Absconditabacteria were detected only in sample S36. Among them, the phylum Firmicutes was more abundant in sample S35 and S36 than in sample S34. Phylum Proteobacteria was also more abundant in sample S34 than in samples S35 and S36. Similarly, phylum Cyanobacteria was more abundant in sample S36 than in samples S34 and S35. However, phyla Synergistetes, Verrucomicrobia and Chloroflexi were the least abundant in sample S34, S35 and S36, respectively (Table 5.19).

Table 5.19**:** Relative abundance of bacterial composition in three traditional fermented food samples at the phylum level

Number	**Phylum**	**Sample**		
		S34	**S35**	**S36**
1.	Firmicutes	0.330829	0.920798	0.92133
2.	Proteobacteria	0.646168	0.059244	0.052929
3.	Cyanobacteria	0.00323	0.005073	0.014446
4.	Actinobacteria	0.002595	0.004763	0.003227
5.	Bacteroidetes	0.008329	0.006276	0.006975
6.	Fusobacteria	0.008407	0.00306	0.000381
7.	Tenericutes	0.000182	0.000524	0.000457
8.	Spirochaetes	3.89E-05	0.000155	0.000152
9.	Saccharibacteria	0.00013	4.76E-05	2.54E-05
10.	Deferribacteres	7.78E-05	2.38E-05	6.35E-05
11.	Verrucomicrobia	0	1.19E-05	0
12.	SR1_(Absconditabacteria)	0	2.38E-05	0
13.	Synergistetes	1.3E-05	0	0
14.	Chloroflexi	0	0	1.27E-05

In the present study, the bacterial communities at the genus level in traditional fermented food products are shown in Figure 5.5. Among the detected genera, the genus *Lactobacillus* was more abundant in samples 35 (73.77%) and 36 (76.01%) than in

sample 34 (17.12%). However, genus *Zymomonas* was more abundant in sample 34 (56.16%) than in samples 35 (2.60%) and 36 (2.71%). On the other hand, samples 35, 34 and 36 were more dominated by genus *Streptococcus* with relative abundance of (4.15%), (2.67%) and (2.32%), respectively (Figure 5.5). Similarly, genus *Leuconostoc* was more dominant in samples 35 (2.93%) and 36 (2.87%) than in sample 34 (2.37%). Genus *Bacillus* was also more abundant in samples S35 (1.95%) and S36 (1.76%) than in sample S34 (1.41%). Likewise, genus *Weissella* was more abundant in samples S35 (1.09%) and S36 (1.06%) than in sample S34 (0.78%). However, others were dominant in sample 34 (11.54%) than in samples 35 (6.64%) and 36 (6.06%). In general, the diversity of bacterial communities between samples and within samples showed a remarkable difference (Figure 5.5).

Figure 5.5**:** Relative abundance of bacterial community composition in fermented food samples at the level of genus

5.5 Discussion

In the present study, an analysis of the 16S rRNA amplicon data for the relative abundance at different taxonomic levels indicate that, the microbial communities of the traditional fermented food samples were complex with organisms belonging to diverse phyla. Among them, Firmicutes was the most abundant phylum, followed by Proteobacteria and *Cyanobacteria*. In accordance with the present study, Singh *et al.* (2019) have reported that among bacteria, Firmicutes (82.31 to 93.99%) was the abundant phylum followed by Actinobacteria (1.05 to 8.87%) and Proteobacteria (1.55 to 8.22%) in *kinema*, a traditional fermented soybean product of Sikkim Himalaya, India. Similarly, The Firmicutes were also identified as the most abundant phylum in traditional starter cultures for enset fermentation from Ethiopia (Addisu Fekadu *et al.*, 2018) and sorghum and millet slurries from Zimbabwe (Gabaza *et al.*, 2019).

In line with this, Liu and Tong (2017) described that at the phylum level, Proteobacteria and Firmicutes were the top two dominant phyla in traditional fermented vegetables in China, accounting for 88.2 to 99.3% of the total phyla. Very recently, Mandhania *et al.* (2019) have also evaluated that bacterial diversity and microbial succession during the fermentation of traditional Indian food (*idli*) at different times of fermentation using 16S rRNA amplicon sequencing. Accordingly, the results highlighted that most of the microbiota belonged to phylum Firmicutes (70%) followed by Proteobacteria (22%). In agreement with this, the predominance of Firmicutes, and the presence of Proteobacteria as minority groups in traditional fermented foods are in consistence with the previous findings on the fermented soybean food products, e.g., *douchi* (Yang *et al.*, 2016).

Moreover, Tesfemariam Berhe *et al.* (2019) have reported the dominance of two phyla in *Dhanaan,* an Ethiopian traditional fermented camel milk samples. Generally, the current results showed that the microbial communities present in traditional fermented food samples were less diverse compared to other studies (Wang *et al.*, 2018). In general, we can therefore conclude that this component of the *teff* dough, *ergo* and *kocho* microbiota is a beneficial component, which could also be a candidate for producing starter culture.

Overall, among the detected bacterial communities, LAB of the traditional fermented food samples hold a promising avenue in food biotechnology Among the identified LAB, the genus *Lactobacillus* was the most abundant organism in traditional fermented food samples followed by the non-LAB genus *Zymomonas*. This is having also been shown during the current *in vitro* study where the genus *Lactobacillus* was the most abundant in traditional fermented foods. These results are in agreement with the previous report, which indicated the dominance of LAB in traditional starter cultures for *enset* fermentation from Ethiopia (Addisu Fekadu *et al.*, 2018). Similarly, previous studies have also reported that LAB were the dominant group during the fermentation of starch-based fermented foods (Adane Hailu *et al.*, 2018; Lu *et al.*, 2008).

Park *et al*. (2012) also indicated that the genera *Lactobacillus*, *Leuconostoc* and *Weisella* were affiliated with fermented vegetables (*kimchi*) throughout the fermentation period. These results are in accordance with the previous report, which indicated the dominance of genus *Lactobacillus* in Chinese sauerkraut fermentation, followed by *Lactococcus* regardless of the inoculated starter culture (Du *et al.*, 2018). Likewise, Mandhania *et al.* (2019) have described that the fermentation of Indian *idli* was primarily driven by lactic

acid bacteria as they constituted 86% of the total bacterial population where the genus *Weissella* emerged as the most important organism in the fermentation process.

In general, these differences in bacterial community may result from the possible influences of manufacturing location and conditions, and/or the type of raw materials used during fermentation. So, analysis of the composition of microbiomes was important to know the bacterial composition of fermented foods in order to assess its safety and to develop starter cultures that mimic the traditional fermentations in order to develop foods with the same characteristics but ensuring the microbiological safety of the foods

5.6 Conclusions

Among the identified bacterial communities, Firmicutes and Proteobacteria were the major dominant phyla in the three traditional fermented food samples. At the genus level, *Lactobacillus* was the top abundant genus in fermented *teff* dough, *ergo* and *kocho* products, whereas the microbiota of fermented ergo was dominated by the genus *Zymomonas*. Generally, these results indicate a high level of bacterial diversity in traditional fermented foods that demonstrates the possible influences of the manufacturing process and/or the type of raw materials used on bacterial community structure. The LAB of the traditional fermented food samples might represent a potential source of starter cultures needed for biotechnological development in food industries.

Chapter 6 General Conclusion and Recommendations

6.1 Conclusion

A wide biodiversity of bacterial communities was identified among the three traditionally Ethiopian fermented food products. Among the identified bacterial communities, Firmicutes and Proteobacteria were the dominant phyla in the three traditional fermented food samples. At the genus level, *Lactobacillus* was the most abundant genus in fermented *teff* dough, *kocho* and ergo, products, whereas *Zymomonas* was the top dominant genus in fermented *ergo*. Generally, the three traditionally Ethiopian fermented food products are rich in potential probiotic lactic acid bacterial strains that may exhibit antimicrobial efficacy against foodborne pathogens due to their bactericidal properties. Accordingly, from these traditional fermented food products, some *Lactobacillus* species and *Lactococcus* species were found to have potentially probiotic characteristics. Among the selected potential probiotic lactic acid bacterial strains, *Lc. lactis* AAUE124, *Lb. paracasei* AAUK114 and *Lb. plantarum* AAUK132 were able to show a protective effect against *Salmonella* Typhimurium DT104 infection in experimental mice.

6.2 Recommendations

- The potential probiotic strains should be used for the production of functional fermented products.
- Further advanced bioinformatics tools should be used to interpret the huge raw sequence data generated.
- In parallel to metagenomics, future studies should also involve a wider study on microbial community of the traditional fermented foods using other functional genomic approaches such as transcriptomic, metabolomics, and proteomics in an effort to develop important food products.
- Further advanced characterization of bacteriocin at gene level should be conducted in order to obtain maximum benefit from this metabolite.

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Appendices

KOH-test (test on lipopolysaccharide)

The KOH test was used to determine the gram reaction of LAB isolates. LAB cultures were grown on MRS agar at 37°C for 24 h under anaerobic conditions. A drop of 3% aqueous KOH was placed on a clean slide. Using a sterile loop, visible cells from fresh cultures were transferred to a drop of 3% KOH. The cells and KOH were mixed thoroughly on the slide and stirred constantly over an area about 1-2 cm^2. The isolates, which did not give a viscid product, were selected since lactic acid bacteria (LAB) are known as Gram-positive cells (Powers, 1995).

Catalase Test

Overnight cultures of isolates were grown on MRS agar at +37°C for 24 h under anaerobic conditions. The catalase test was conducted by dripping two drops of hydrogen peroxide (3%) on 24 h old cultures on a glass slide. Catalase test shows a positive reaction that is characterized by the formation of oxygen bubbles. Therefore, the isolates that did not give gas bubbles, were selected for subsequent activities.

Spore Staining

Gram-positive and catalase-negative isolates were grown on MRS agar at +37°C for 24 h under anaerobic conditions. The spore-staining procedures were carried out following the standard methods (Prescott *et al.*, 2002). After the spore-staining technique, endospore formation was examined under light microscopy using oil immersion objectives (Motic Microscopes, B3-220ASC, and EUROPEAN Division). The isolates which did not form endospores were selected for further analysis.

Appendix 1**:** Preliminary screening of lactic acid bacteria isolates from fermented food products.

Number	Isolate Code	Source	KOH test	Catalase test	Spore staining
1.	T006	Teff dough	-	-	-
2.	T008	Teff dough	-	-	-
3.	T009	Teff dough	-	-	-
4.	T010	Teff dough	-	-	-
5.	T011	Teff dough	-	-	-
6.	T012	Teff dough	-	-	-
7.	T015	Teff dough	-	-	-
8.	T018	Teff dough	-	-	-
9.	T020	Teff dough	-	-	-
10.	T022	Teff dough	-	-	-
11.	T025	Teff dough	-	-	-
12.	T028	Teff dough	-	-	-
13.	T029	Teff dough	-	-	-
14.	T031	Teff dough	-	-	-
15.	T033	Teff dough	-	-	-
16.	T035	Teff dough	-	-	-
17.	T036	Teff dough	-	-	-
18.	T038	Teff dough	-	-	-
19.	T039	Teff dough	-	-	-
20.	T040	Teff dough	-	-	-
21.	T042	Teff dough	-	-	-
22.	T043	Teff dough	-	-	-
23.	T044	Teff dough	-	-	-
24.	T045	Teff dough	-	-	-
25.	T046	Teff dough	-	-	-
26.	T047	Teff dough	-	-	-
27.	T048	Teff dough	-	-	-
28.	T050	Teff dough	-	-	-
29.	T052	Teff dough	-	-	-
30.	T054	Teff dough	-	-	-
31.	T055	Teff dough	-	-	-
32.	T058	Teff dough	-	-	-
33.	T060	Teff dough	-	-	-
34.	T062	Teff dough	-	-	-
35.	T063	Teff dough	-	-	-
36.	T064	Teff dough	-	-	-
37.	T065	Teff dough	-	-	-
38.	T067	Teff dough	-	-	-
39.	T068	Teff dough	-	-	-
40.	T069	Teff dough	-	-	-

41.	T070	Teff dough	-	-	-
42.	T071	Teff dough	-	-	-
43.	T072	Teff dough	-	-	-
44.	T073	Teff dough	-	-	-
45.	T075	Teff dough	-	-	-
46.	T076	Teff dough	-	-	-
47.	T078	Teff dough	-	-	-
48.	T079	Teff dough	-	-	-
49.	T080	Teff dough	-	-	-
50.	T081	Teff dough	-	-	-
51.	T082	Teff dough	-	-	-
52.	T084	Teff dough	-	-	-
53.	T085	Teff dough	-	-	-
54.	T086	Teff dough	-	-	-
55.	T088	Teff dough	-	-	-
56.	T090	Teff dough	-	-	-
57.	T091	Teff dough	-	-	-
58.	T093	Teff dough	-	-	-
59.	T094	Teff dough	-	-	-
60.	T096	Teff dough	-	-	-
61.	T097	Teff dough	-	-	-
62.	T098	Teff dough	-	-	-
63.	T100	Teff dough	-	-	-
64.	T101	Teff dough	-	-	-
65.	T102	Teff dough	-	-	-
66.	T104	Teff dough	-	-	-
67.	T105	Teff dough	-	-	-
68.	T108	Teff dough	-	-	-
69.	T110	Teff dough	-	-	-
70.	T111	Teff dough	-	-	-
71.	T112	Teff dough	-	-	-
72.	T113	Teff dough	-	-	-
73.	T115	Teff dough	-	-	-
74.	T117	Teff dough	-	-	-
75.	T118	Teff dough	-	-	-
76.	T119	Teff dough	-	-	-
77.	T120	Teff dough	-	-	-
78.	T121	Teff dough	-	-	-
79.	T122	Teff dough	-	-	-
80.	T123	Teff dough	-	-	-
81.	T125	Teff dough	-	-	-
82.	T126	Teff dough	-	-	-
83.	T127	Teff dough	-	-	-
84.	T128	Teff dough	-	-	-

85.	T129	Teff dough	-	-	-
86.	T130	Teff dough	-	-	-
87.	T131	Teff dough	-	-	-
88.	T132	Teff dough	-	-	-
89.	T134	Teff dough	-	-	-
90.	T135	Teff dough	-	-	-
91.	T136	Teff dough	-	-	-
92.	T137	Teff dough	-	-	-
93.	T139	Teff dough	-	-	-
94.	T140	Teff dough	-	-	-
95.	T142	Teff dough	-	-	-
96.	T145	Teff dough	-	-	-
97.	T146	Teff dough	-	-	-
98.	T147	Teff dough	-	-	-
99.	T148	Teff dough	-	-	-
100.	T149	Teff dough	-	-	-
101.	T150	Teff dough	-	-	-
102.	E004	Ergo	-	-	-
103.	E009	Ergo			
104.	E012	Ergo	-	-	-
105.	E018	Ergo	-	-	-
106.	E021	Ergo	-	-	-
107.	E024	Ergo	-	-	-
108.	E026	Ergo	-	-	-
109.	E027	Ergo	-	-	-
110.	E030	Ergo	-	-	-
111.	E031	Ergo	-	-	-
112.	E035	Ergo	-	-	-
113.	E036	Ergo	-	-	-
114.	E039	Ergo	-	-	-
115.	E040	Ergo	-	-	-
116.	E042	Ergo	-	-	-
117.	E043	Ergo	-	-	-
118.	E045	Ergo	-	-	-
119.	E047	Ergo	-	-	-
120.	E048	Ergo	-	-	-
121.	E050	Ergo	-	-	-
122.	E051	Ergo	-	-	-
123.	E052	Ergo	-	-	-
124.	E054	Ergo	-	-	-
125.	E056	Ergo	-	-	-
126.	E057	Ergo	-	-	-
127.	E058	Ergo	-	-	-
128.	E060	Ergo	-	-	-

129.	E062	Ergo	-	-	-
130.	E063	Ergo	-	-	-
131.	E069	Ergo	-	-	-
132.	E074	Ergo	-	-	-
133.	E076	Ergo	-	-	-
134.	E077	Ergo	-	-	-
135.	E079	Ergo	-	-	-
136.	E080	Ergo	-	-	-
137.	E081	Ergo	-	-	-
138.	E082	Ergo	-	-	-
139.	E083	Ergo	-	-	-
140.	E084	Ergo	-	-	-
141.	E086	Ergo	-	-	-
142.	E087	Ergo	-	-	-
143.	E088	Ergo	-	-	-
144.	E090	Ergo	-	-	-
145.	E093	Ergo	-	-	-
146.	E094	Ergo	-	-	-
147.	E095	Ergo	-	-	-
148.	E096	Ergo	-	-	-
149.	E098	Ergo	-	-	-
150.	E100	Ergo	-	-	-
151.	E111	Ergo	-	-	-
152.	E112	Ergo	-	-	-
153.	E113	Ergo	-	-	-
154.	E116	Ergo	-	-	-
155.	E117	Ergo	-	-	-
156.	E118	Ergo	-	-	-
157.	E119	Ergo	-	-	-
158.	E120	Ergo	-	-	-
159.	E121	Ergo	-	-	-
160.	E122	Ergo	-	-	-
161.	E124	Ergo	-	-	-
162.	E125	Ergo	-	-	-
163.	E127	Ergo	-	-	-
164.	E128	Ergo	-	-	-
165.	E129	Ergo	-	-	-
166.	E130	Ergo	-	-	-
167.	E131	Ergo	-	-	-
168.	E132	Ergo	-	-	-
169.	E133	Ergo	-	-	-
170.	E136	Ergo	-	-	-
171.	E137	Ergo	-	-	-
172.	E138	Ergo	-	-	-

173.	E139	Ergo	-	-	-
174.	E140	Ergo	-	-	-
175.	E141	Ergo	-	-	-
176.	E142	Ergo	-	-	-
177.	E143	Ergo	-	-	-
178.	E144	Ergo	-	-	-
179.	E145	Ergo	-	-	-
180.	E146	Ergo	-	-	-
181.	E148	Ergo	-	-	-
182.	E149	Ergo	-	-	-
183.	K011	Kocho	-	-	-
184.	K012	Kocho	-	-	-
185.	K013	Kocho	-	-	-
186.	K018	Kocho	-	-	-
187.	K020	Kocho	-	-	-
188.	K022	Kocho	-	-	-
189.	K023	Kocho	-	-	-
190.	K025	Kocho	-	-	-
191.	K029	Kocho	-	-	-
192.	K030	Kocho	-	-	-
193.	K031	Kocho	-	-	-
194.	K033	Kocho	-	-	-
195.	K034	Kocho	-	-	-
196.	K036	Kocho	-	-	-
197.	K038	Kocho	-	-	-
198.	K039	Kocho	-	-	-
199.	K040	Kocho	-	-	-
200.	K042	Kocho	-	-	-
201.	K045	Kocho	-	-	-
202.	K046	Kocho	-	-	-
203.	K047	Kocho	-	-	-
204.	K050	Kocho	-	-	-
205.	K051	Kocho	-	-	-
206.	K054	Kocho	-	-	-
207.	K055	Kocho	-	-	-
208.	K056	Kocho	-	-	-
209.	K058	Kocho	-	-	-
210.	K059	Kocho	-	-	-
211.	K061	Kocho	-	-	-
212.	K062	Kocho	-	-	-
213.	K063	Kocho	-	-	-
214.	K064	Kocho	-	-	-
215.	K065	Kocho	-	-	-
216.	K066	Kocho	-	-	-

217.	K068	Kocho	-	-	-
218.	K070	Kocho	-	-	-
219.	K071	Kocho	-	-	-
220.	K072	Kocho	-	-	-
221.	K074	Kocho	-	-	-
222.	K076	Kocho	-	-	-
223.	K077	Kocho	-	-	-
224.	K080	Kocho	-	-	-
225.	K082	Kocho	-	-	-
226.	K083	Kocho	-	-	-
227.	K085	Kocho	-	-	-
228.	K086	Kocho	-	-	-
229.	K089	Kocho	-	-	-
230.	K090	Kocho	-	-	-
231.	K091	Kocho	-	-	-
232.	K092	Kocho	-	-	-
233.	K094	Kocho	-	-	-
234.	K096	Kocho	-	-	-
235.	K097	Kocho	-	-	-
236.	K099	Kocho	-	-	-
237.	K102	Kocho	-	-	-
238.	K105	Kocho	-	-	-
239.	K108	Kocho	-	-	-
240.	K109	Kocho	-	-	-
241.	K112	Kocho	-	-	-
242.	K114	Kocho	-	-	-
243.	K115	Kocho	-	-	-
244.	K116	Kocho	-	-	-
245.	K118	Kocho	-	-	-
246.	K120	Kocho	-	-	-
247.	K122	Kocho	-	-	-
248.	K123	Kocho	-	-	-
249.	K124	Kocho	-	-	-
250.	K125	Kocho	-	-	-
251.	K127	Kocho	-	-	-
252.	K130	Kocho	-	-	-
253.	K131	Kocho	-	-	-
254.	K132	Kocho	-	-	-
255.	K135	Kocho	-	-	-
256.	K138	Kocho	-	-	-
257.	K145	Kocho	-	-	-
258.	K146	Kocho	-	-	-
259.	K147	Kocho	-	-	-
260.	K148	Kocho	-	-	-

Appendix 2:Modified MRS broth for testing the growth of LAB at different temperatures

Ingredients	**g/L**
Peptone	10.0
Meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1ml
K2HPO4	2
Sodium acetate	5.0
Triammonium citrate	2.0
MgSO4.7H2O	0.2
MnSO4.4H2O	0.05
Bromoecresol purple	0.04
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and sterilized by autoclaving at 121°C for 15 min.

Appendix 3:Modified MRS broth for the growth of LAB at different NaCl concentrations

Ingredients	**g/L**
Peptone	10.0
Meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1ml
K2HPO4	2
Sodium acetate	5.0
Triammonium citrate	2.0
MgSO4.7H2O	0.2
MnSO4.4H2O	0.05
Bromoecresol purple	0.04
NaCl	For the concentration of 4% and 6.5%
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and sterilized by autoclaving at 121°C for 15 min.

Appendix 4: Modified MRS broth for testing ammonia production from arginine

Ingredients	g/L
Peptone	10.0
Meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1ml
K2HPO4	2
Sodium acetate	5.0
Triammonium citrate	2.0
MgSO4.7H2O	0.2
MnSO4.4H2O	0.05
Arginine	1.5
Deionized water	1000ml

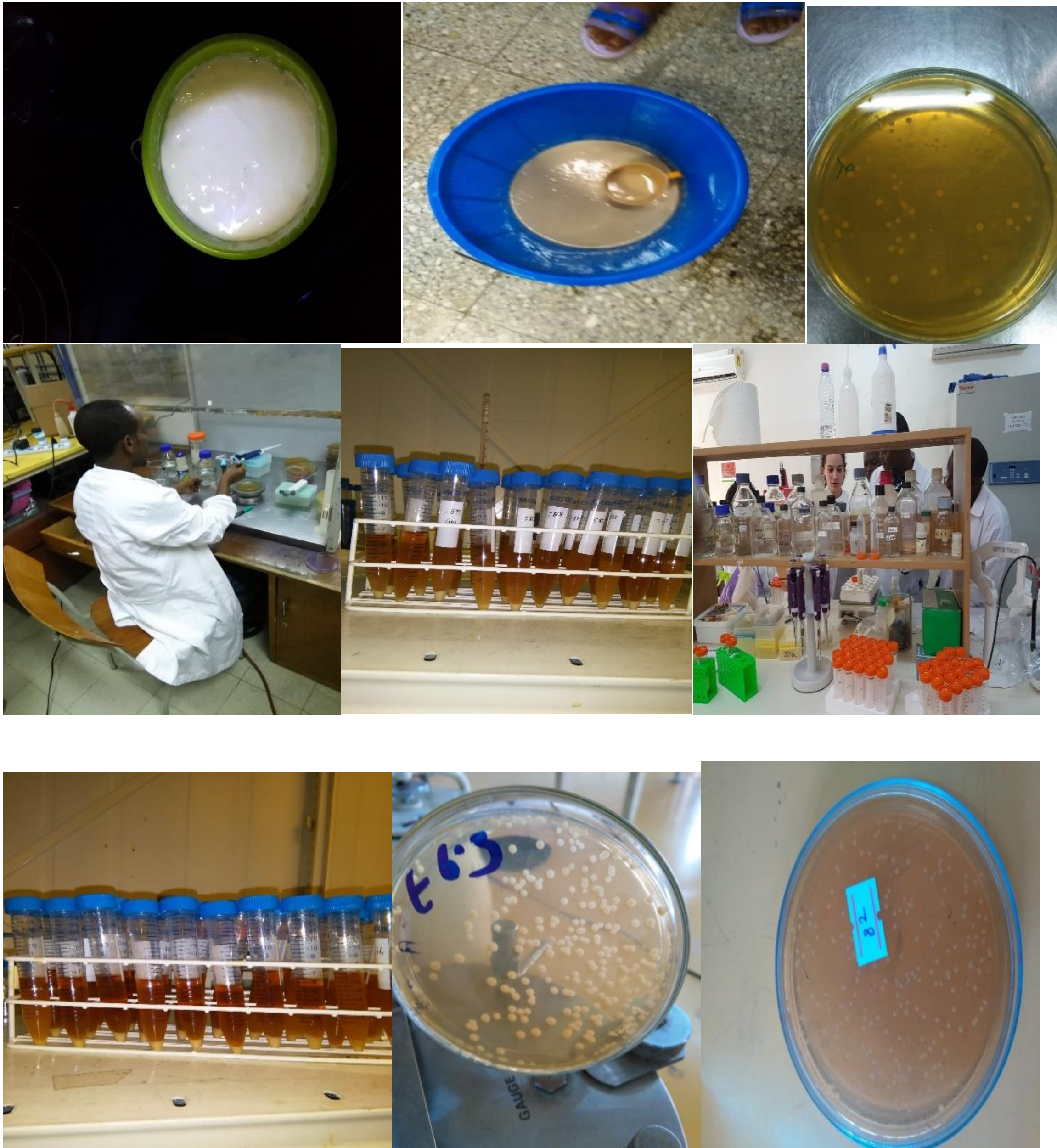
All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and sterilized by autoclaving at 121°C for 15 min.

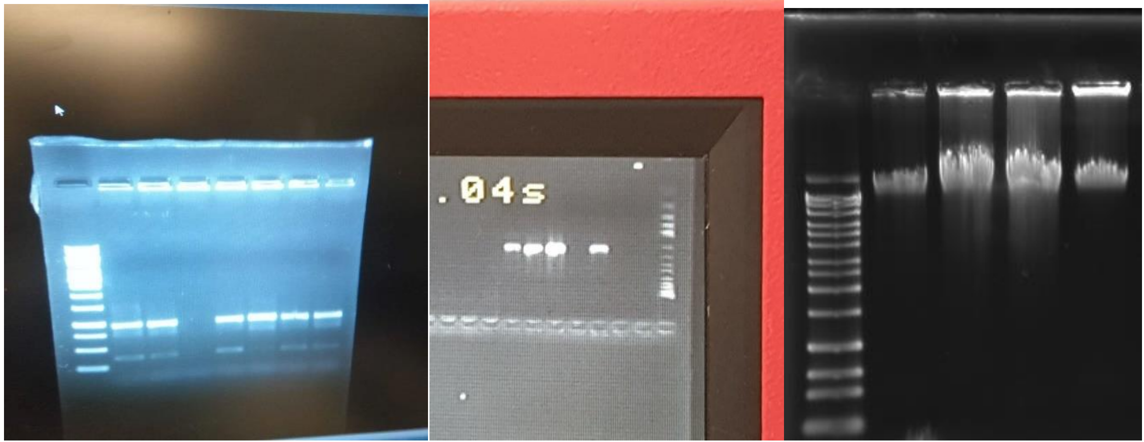
Appendix 5: Modified MRS broth for testing gas production from glucose

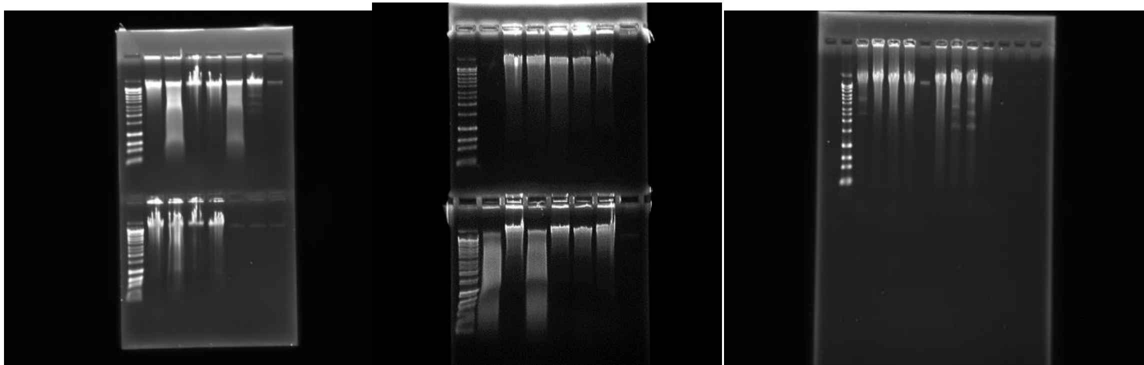
Ingredients	g/L
Peptone	10.0
Meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1ml
K2HPO4	2
Sodium acetate	5.0
MgSO4.7H2O	0.2
MnSO4.4H2O	0.05
Bromoecresol purple	0.04
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and inverted Durham tubes were distributed to each test tube, and lastly sterilized by autoclaving at 121°C for 15 min.

Appendix 6**:** Microbial analysis of traditionally Ethiopian fermented food products



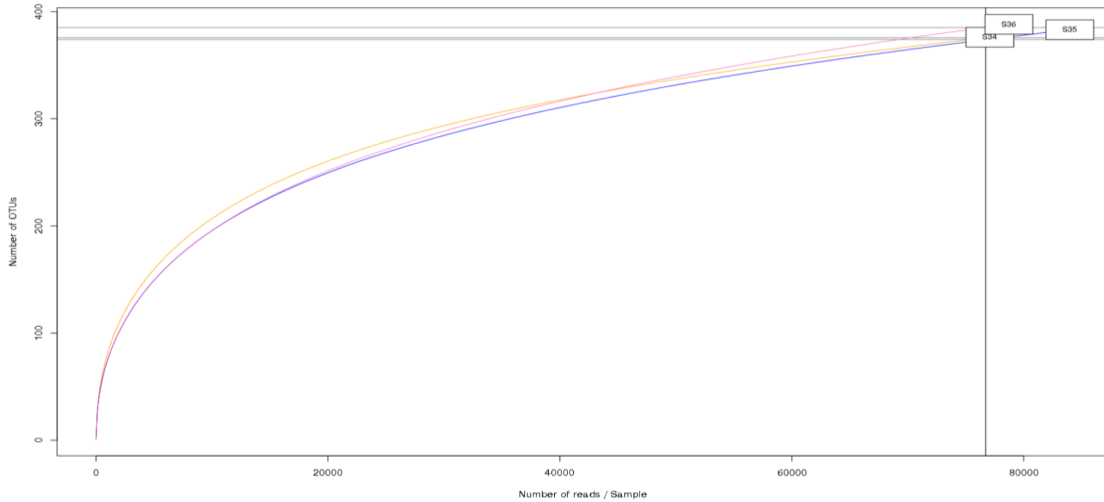

.04s



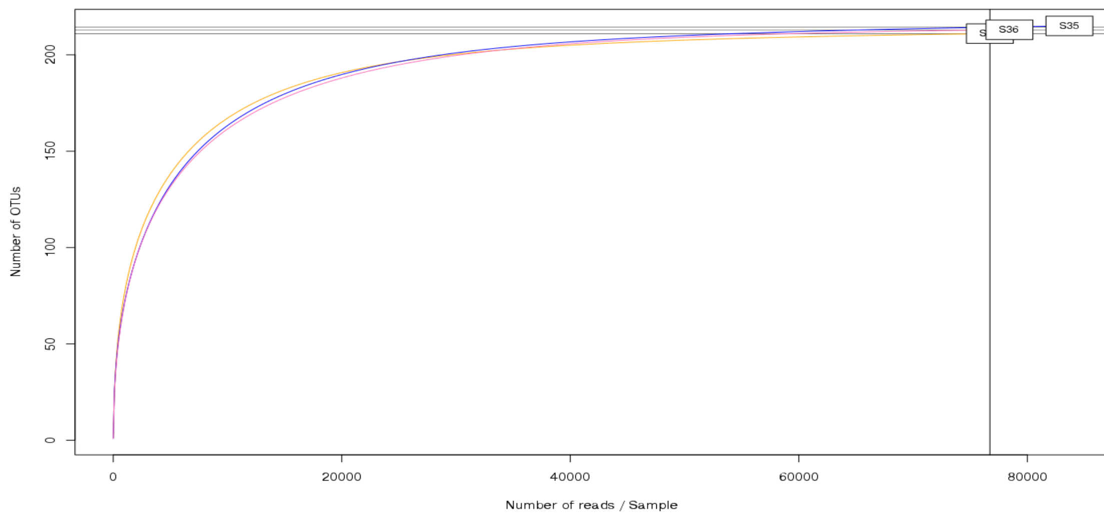
P+S
challenged

Appendix 7**:** Microbial community analysis using bioinformatics

Before filtering rare OTU

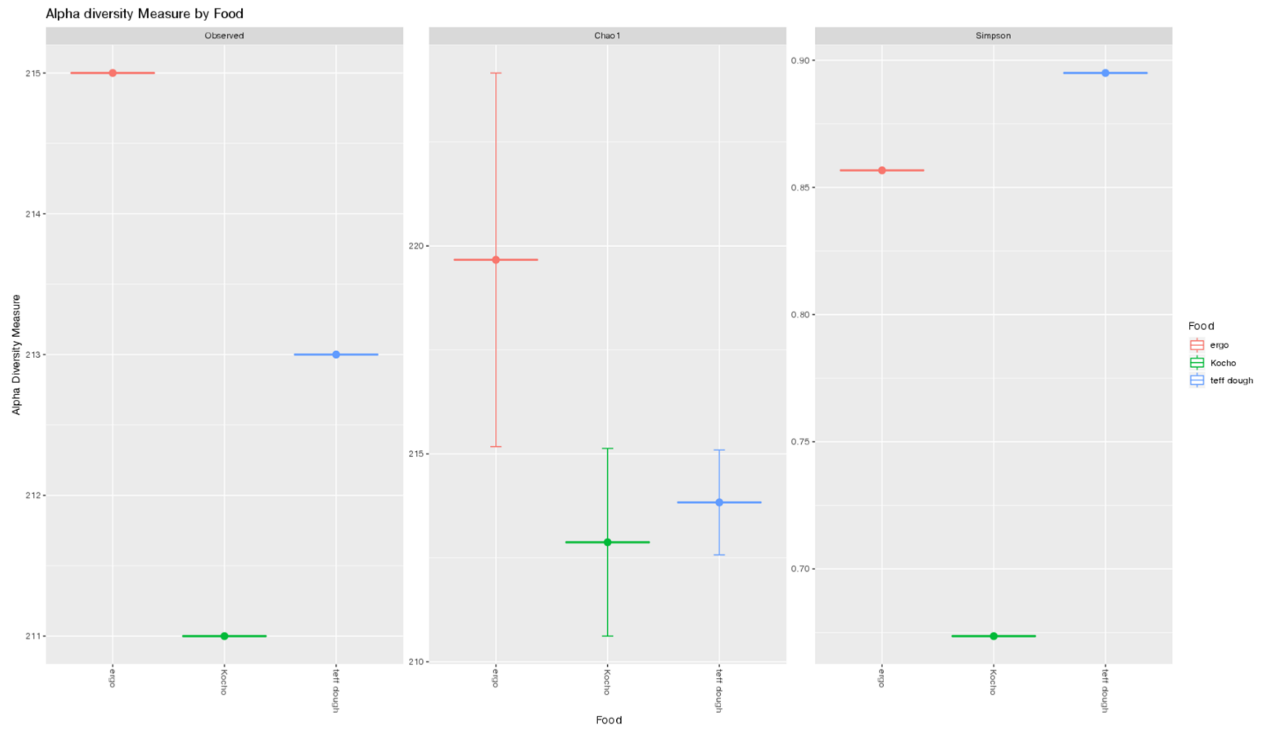


After filtering rare OTU



Before filtering rare OTUs the rarefaction curve was increasing and did not clearly converge. Hence, after removing the low abundant OTUs, the curve of all samples gradually reached a horizontal asymptote to the right of the curve. This shape attests that we have enough depth of sequencing. The vertical line indicates the minimum number of reads. All samples exceed this threshold. Thus, all samples can be included in the downstream analysis.

Alpha diversity



p-value = 0.3679 p-value = 0.3679 p-value = 0.3679

Beta diversity