CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF LACTIC ACID BACTERIA ISOLATED FROM *SHIMPIA* AGAINST SELECTED PATHOGENIC MICROORGANISMS

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A THESIS SUBMITTED TO THE SCHOOL OF ANIMAL AND RANGE SCIENCES, COLLEGE OF AGRICULTURE, SCHOOL OF GRADUATE STUDIES, HAWASSA UNIVERSITY

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APPROVAL SHEET – 1

This is to certify that the thesis entitled "Study On characterization and antimicrobial activity of lactic acid bacteria isolated from *Shimpia* (an Ethiopian traditional fermented milk product), against selected pathogenic microorganisms" submitted in partial fulfillment of the requirements for the degree of Master of Science in Animal and Range Sciences with a specialization of Dairy Science & Technology of the Graduate Program of the School of Animal and Range Sciences, Hawassa University, College of Agriculture and is a record of original research carried out by Yimer Teketay, under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

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APPROVAL SHEET – 2

We, the undersigned, members of the Board of Examiners of the final open defence by Yimer Teketay have read and evaluated the thesis entitled ‘'Study On characterization and antimicrobial activity of lactic acid bacteria isolated from *Shimpia* (an Ethiopian traditional fermented milk product), against selected pathogenic microorganisms” and examined the candidate. This is therefore to certify that the thesis has been accepted in partial fulfilment of the requirements for the degree of Masters of Science.

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Final approval and acceptance of the thesis is contingent upon the submission of the final copy of the thesis to the School of Graduate Council (SGC) of the candidate's major department.

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STATEMENT OF THE AUTHOR

I declare that this thesis is my actual work and that all sources of materials used for this thesis have been drearily recognized. This thesis has been submitted in partial fulfillment of the requirements for Master of Science at the Hawassa University and is deposited at the University Library to be made available to borrowers under rules of the Library. I honestly state that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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Place: College of Agriculture, Hawassa University, Hawassa

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2 LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
CFU	Colony Forming Unit
EUCAST	European Committee for Antimicrobial Susceptibility Testing
IDF	International Dairy Federation
TBC	Total bacteria count
TS	Total solid
VRBA	Violet Red Bile Agar
YMC	Yeast and Mould Count
LAB	Lactic acid bacteria

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CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF LACTIC ACID BACTERIA ISOLATED FROM *SHIMPIA* AGAINST SELECTED PATHOGENIC MICROORGANISMS

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ABSTRACT

Traditional dairy products are food products prepared by traditional knowledge of source community. They can have different physicho-chemical and microbiological properties based on the milk source, way of processing and agro-ecological factors. The aim of this study was to evaluating physico-chemical and microbiological quality of shimpia and characterization and testing the antibacterial potential of lactic acid bacteria isolates from Shimpia in the selected districts of Wolayta zone, southern Ethiopia. In line with this, a traditional fermented milk product produced among the Wolayta community called Shimpia was studied. The study was conducted in three selected agro-ecologically different kebeles. Thus, for the questionnaire based survey study, a total of 162 farmers with Kokatemarachere (79), Bossakecha (45) and Gulgula (38) were participated. Additionally, 10 Shimpia sample from each kebele with a total of 30 samples were collected to determine the physicho-chemical and microbial analysis using standard procedures. Besides, the antimicrobial activities of LAB isolates from Shimpia were tested against selected pathogens using disc diffusion assay. The results showed 57.9% of respondents produced Shimpia from milk sourced from local breed cows. The majority (64.8%) of the respondents used tap water for cleaning of equipments used for Shimpia production. Nearly half of (52.5%) the respondents used locally available herbs as cleaning agent. The pH of Shimpia prepared at farmers house ranged from 4.5 to 4.7 with average constituents of total solid 11.7%, fat 4.5%, protein 3.1%, lactose 3.7% and ash 0.7%. The total solid and fat content of the samples were significantly different across the agro-ecological locations ($P< 0.05$). In addition, enumeration of microorganisms (cfu/g) showed that total aerobic bacteria count (8.79±0.04), yeast and mould count (6.49±0.05), total lactic acid bacteria, (4.77±0.01), and total Coliform count (4.22±0.03). A total of 72 strains of LAB belonging to Lactobacillus, Streptococcus, Pediococcus, Leuconostoc, Lactococus and Entrococus species were isolated and its antimicrobial activities was tested against Salmonella typhi, Listeria monocytogenes, S,aurious (ATCC-25923) and E.coli O157:H7(ATCC-25922). However, only 8 strains of LAB showed antagonistic effect against pathogens with inhibition zone ranged from 9.0mm to 11.8mm. The isolates' antagonism effect against different pathogens was significantly different at $P < 0.05$.

Key Words: Shimpia, lactic acid bacteria, antimicrobial activity, traditional fermented milk product, Ethiopia

1. INTRODUCTION

1.1. Background

Microorganisms have played an important role in food fermentation process and preservation history. Fermented food production mostly used Lactic acid bacteria (LAB) which contributes to flavor development as well as safe metabolic activities (Anteneh *et al.*, 2011). During fermentation; they utilize available sugar for the production of organic acids and other metabolites. In common fermented milk products such as yoghurt LAB have an important role in the inhibition of food borne pathogenic and spoilage microorganisms since they produce antimicrobial metabolites that include lactic acid, acetic acid, and other organic acids, hydrogen peroxide, and other substances like bacteriocin (Abdulkadir *et al*, 2011).

The rural communities in Ethiopia produce fermented milk by traditional methods. The major fermented milk products produced by small-holder farmers include "*Ergo*" (fermented sour milk), "*Ititu*" (Fermented milk curd), "*Kibe*" (traditional butter), "*Neterkibe*" (spiced and clarified ghee), "*Ayib*" (cottage cheese), "*Arerra*" (Sour defatted milk), "*Aguat*" (whey), and *Shimpia* (fermented milkshake). *Shimpia* is a traditional naturally fermented milk product, which is unique for the *Wolayta* community and has some resemblance to *Ergo*. It is thick, smooth, of uniform appearance, and usually has a white milky color when prepared carefully. The product is prepared by 10-15 minutes churning of naturally fermented *Ergo* curd until it forms foam on the verge of producing fine butter grains. During churning of milk curdle, fat globule membranes braked down and fatty acids diffuse through the curdle shake and this gives *shimpia* unique a pleasant odor and creamy taste.

In Ethiopia, some studies have been carried out on the isolation, characterization, and antimicrobial activity of LAB from traditional fermented milk products. Eyassu. (2012) reported

a total of six species of LAB isolated and characterized from *Ititu,* Ethiopian traditional fermented camel milk and were grouped under three genera, Lactobacillus (85), Lactococcus (36), and Enterococcus (25). The isolated species of lactic acid bacteria were *Lactobacillus plantarum* (32%), *Lactobacillus delbrueckii subsp. bulgaricus* (17%), *Lactobacillus salivarius* (9%), *Lactococcus lactis subsp. lactis* (18%), *Lactococcus lactis subsp. cremoris* (7%) and *Enterococcus faecalis* (17%). Regarding antimicrobial activities of lactic acid bacteria, Mekonen and Mogesie **(**2005) studied fate of Escherichia coli O157:H7 during the processing and storage of '*Ergo'* and '*Ayib'*, and they indicated the decrease in the population of the *E.coli* O157:H7 and resulted in low pH during the course of fermentation due to its antagonistic activities of the LAB.

Shimpia is being used as healthy traditional milk-origin food by the Wolayta community for many years. Since *Shimpia* is produced from unpasteurized milk, pathogenic bacteria may have a great chance to grow in it. *Escherircia coli salmonella, listeria monocytogenes,* and *staphylococus aureus* are among the most expected pathogenic bacteria in raw milk in Ethiopia. So the fate of these pathogens during fermentation of raw milk for *Shimpia* preparation should be studied. Due to this, studying important LAB available in *Shimpia* is important to show its potential for future economic use as a bio preservative in the food processing industry. Yet, there is no research done on *Shimpia* in this regard and important fermentative microbes in it. This study is therefore aimed to generate information on the traditional processing methods, physicho-chemical properties, microbiological quality, characteristics of certain LAB in *Shimpia,* and their potential to inhibit the growth of undesirable bacteria.

1.2. Objectives

1.2.1. General Objectives

The overall aim of this study was to evaluating physico-chemical and microbiological quality of *Shimpia* and characterization and testing the antibacterial potential of lactic acid bacteria isolates from *Shimpia*, traditional fermented milk in *Wolayta*, southern Ethiopia.

1.2.2. Specific objectives

The specific objectives include:

1. To asses traditional processing techniques of *Shimpia*
2. To evaluate the physico-chemical and microbiological quality of *Shimpia*
3. To isolate and characterize lactic acid bacteria from *Shimpia*
4. To evaluate the antibacterial activity of lactic acid bacteria isolated *from Shimpia a*gainst selected pathogens

2. LITERATURE REVIEW

2.1. History of Food Fermentation

The word fermentation is derived from the Latin verb '*fevere'* which means "to boil" and fermentation was defined by Louis Pasteur as "La vie sans lair" (life without air) (Bourdichon *et al.,* 2012). Food fermentation has a long history since ancient times which involves chemical transformation of complex organic compounds into simpler compounds by the action of enzymes, organic catalysts produced by microorganisms including yeast, moulds, and bacteria (Corma, Iborra, and Velty, 2007). Fermentation is a biotechnological process traditionally used as a means of food preservation and evidences have shown that rice, honey, and fruit beverages were produced using fermentation as far back as 7000 B.C in China (Marsh *et al.*, 2014).

The relation between fermented food and health dates back from Neolithic Chinese to ancient Roman era and the earliest evidence suggests that fermentation was an integral part of the old civilization. The production of fermented dairy-based products are mentioned in ancient Sanskrit and Christian scripts, while Romans were the first who revealed the recipe of fermented milk preparation at around 1900 BC (Ray and Roy, 2014).

Fermentation processes have been developed for the production of a wide range of products from chemically simple compounds, *e.g.* ethanol to highly complex macromolecules, *e.g.* polysaccharides. Recently, fermentation technique has been applied to the production and extraction of bioactive compounds in the food, chemical and pharmaceutical industries (Shikha, 2016). Novel fermentation technologies can assist food processors to meet both consumer demands for higher quality and safer products and also the industry demand for energy efficient processes (Pereira and Vicente, 2010). The modern fermentation industry is highly competitive and innovative and has been at the forefront in assessing the potential of new technologies to

improve fermentation processes and yield better quality products. The food fermentation industry requires novel techniques to improve the productivity and quality of fermented products along with new products from a range of food sources (Shikha, 2016).

2.2. Relevance of Fermented Food Products

Fermented food products are currently experienced by every cultural society in the world according to the availability of the food substrate and their food consumption patterns. In many cases, such products play an important role in ethnic identity and culinary enjoyment (Hui *et al*., 2004). For instance, Europe produces the largest quantity of fermented dairy products while Africa is the largest producer of fermented starch crops and legumes-based food products. Similarly, fermented fish products are very common in the south and south-east Asia whereas North America is presumably the biggest producer of fermented beverages and meat products (Khem, 2009).

Over the centuries, fermentation techniques and procedures have evolved, refined, and extended which helped some fermented products such as bread, cheese, and yoghurt to be produced all over the world. Fermentation is commonly used in the food and functional food industry, and there are approximately 5000 varieties of fermented foods and beverages consumed worldwide (Tamang and Kailasapathy, 2010). Fermented food products include those derived from meat (sausages, salami), dairy (yoghurt, cheese, and kefir), soy (natto, miso), fruits (wine), cereals (Bread), vegetables (sauerkraut) and fish (surimi). The secondary metabolites produced during fermentation processes range from antibiotics to peptides and are also referred to as bioactive compounds due to their biological activities which are numerous and range from the prevention of chronic diseases such as diabetes and cardiovascular disease to cancer prevention (Limón *et al.*, 2015).

Bioactive compounds obtained as a result of the fermentation process not only improve the nutritional value of food but also allow shelf-life extension while improving safety profile ie. Consumers are preferring foods that have beneficial components towards health and wellness. As an important aspect of this trend, probiotic fermented foods are getting more attention because of their image as a gut health booster. The increased demand for traditional and/or novel value added fermented products have brought new challenges to the market to develop novel products (Shikha, 2016).

2.3. Fermented Dairy Products

Consumption of fermented milk products, as a healthy and nutritious food, has a long tradition. Initially, the function of milk fermentation was to extend the shelf life of the product and additionally many benefits are achieved, such as improving the digestibility and flavor, as well as the ability to produce a wide range of different products. The fermentation process involved unpredictable and slow souring of milk caused by the indigenous microflora in the milk. However, applications of modern microbiological processes have resulted in the production of various fermented dairy products under controlled conditions (Shikha, 2016). Currently, around 400 different names are applied for traditionally and industrially produced fermented milk products. The specificity of each type of product is reflected by the applied type of microorganisms, milk, and process conditions (Surono and Hosono, 2011).

2.4. Diversity of Fermented Dairy Products

Fermentation of milk is one of the oldest methods for food preservation dated from some 10–15,000 years B.C. It is likely that the origin of yoghurt was the Middle East (Surono and Hosono, 2011). Nowadays, there are numerous types of fermented milk in different parts of the world. On

the basis of the starter culture applied, fermented dairy products could be classified into the following types:

1. Products of lactic fermentation where strains of mesophilic or thermophilic lactic acid bacteria (LAB) are used (yoghurt, zabadi, labneh, acidophilus milk, bio grade, bifighurt, ABT, cultured buttermilk, ymer, dahi, film jolk, tatmjolk, yakult, etc.)
2. Products obtained via alcohol-lactic fermentation (kefir, kumis, acidophilus yeast milk, skyr).
3. Products where molds grow in addition to microorganisms from fermentation of type 1 and 2 are present (Surono and Hosono, 2011).

2.4.1. Kefir

Kefir is a fermented milk beverage originated in the Caucasus region, but it is consumed throughout the world. Kefir grains are cluster of microorganisms held together by a polysaccharide matrix referred to as kefiran, serve as the fermentation inoculums. These grains are small, irregularly shaped, yellowish-white, hard granules that resemble miniature cauliflower blossoms. Kefir is a probiotic food (Otles and Cagindi 2003; Guedes *et al.* 2014), and it is well known that probiotic foods are associated with health benefits that are of significant current interest of food industry (Ahmed *et al*., 2013).

Kefir grains are an example of symbiosis between yeast and bacteria. A variety of yeast and bacteria species that form the kefir grains have been isolated and identified (Puerari*et al.*,2012 and Gao *et al.,* 2013). The *Lactobacillus* genus is the most common genus during the fermentation period. Other lactic bacteria such as species from the *Leuconostoc, Lactococcus,* and *Streptococcus* genera are also commonly detected (Miguel *et al.,* 2010 and Magalhães *et al.,*

2011c),as well as Gram-negative bacteria in the Acetobacter genera (Puerari *et al*. 2012 and Gao *et al*., 2013).

The yeast isolates include species from the following genera: *Kluyveromyces, Candida, Torulaspora, Pichia, Cryptococcus, Debaromyces, Kazachstania,Lachancea, Torulaspora, Zygosaccharomyces, Trichosporon,* and *Saccharomyces* (Garofalo *et al*., 2015). The microorganisms present in kefir perform three types of fermentation: lactic, alcoholic, and acetic (Magalhães *et al*., 2011a, 2011b). Kefir grains are microbial rich, cauliflower-like structures typically consisting of three groups of microorganisms living in symbiotic association. These include lactic acid bacteria (LAB), acetic acid bacteria (AAB), and yeasts (Magalhães *et al*., 2010; Puerari *et al*., 2012 and Garofalo *et al*., 2015).

4.1.1 Kefir Flavor

Lactic acid is the major acid produced during kefir fermentation. Carbonyl compounds (acetaldehyde, ethanol, diacetyl, acetoin, 2-butanone, and ethyl acetate), volatile organic acids (formic, acetic, propionic, and butyric) and nonvolatile acids (lactic, pyruvic, oxalic, and succinic) are the secondary metabolites and are classified as flavor-forming compounds (Magalhães *et al*., 2011 and Puerari *et al*., 2012).

Variations in the storage conditions, growth medium, and environment can affect the development of a community of microorganisms that contribute to the characteristics of the grains (Schoever and Britz, 2003). Therefore, the organoleptic features of kefir are directly linked to the microorganisms present in the grains and can be influenced by changes in the intrinsic factors of the grains (grain activity, grain-to-milk ratio, and starter) or by one or several environment factors such as the incubation temperature, effect of pH, and storage conditions (Otles and Cagindi, 2003).

2.4.2. *Ergo* (traditional sour milk)

Ergo is a traditional naturally fermented milk product, which has some resemblance to yogurt. It is thick, smooth and of uniform appearance and usually has a white milk color when prepared carefully. The product is semi solid and has a pleasant odor and taste. It constitutes a primary sour milk product from which other products may be processed. Depending on the storage temperature, it can be stored for 15-20 days (Almaz, 2001).Ergo is mainly produced by women and they may further process in to more stable products which may be sold in the market, and thus generates income by which other household items may be purchased (Lemma, 2004).

Fermentation is carried out by lactic acid bacteria belonging to the genera *Lactococcus, Streptococcus, Luconostoc,* and *Lactobacillus*. They also observed that *Micrococcus*, coliforms and spore-formers were also present in fairly high numbers during the first 12-14 hr of fermentation. Their population decreased substantial thereafter, which implies an antimicrobial activity besides low pH in the fermented milk (Almaz *et al.,* 1999). Ergo' is produced under non-hygienic environment with a high possibility of contamination with desirable, pathogenic, non-pathogenic and/or spoilage bacteria, the lactic acid bacteria fermentation can reduce the risk of spoilage and pathogenesis (Desalegn, 2013).

Although fermented food products are usually considered safe because of the antagonistic effect of LAB, some food borne pathogens have been reported to survive and grow in fermented milks (Ashenafi, 1992, 1994). Researchers have also proved the survival of *L. monocytogenes*, *Salmonella spp*. *Staphylococcus aureus* and *Bacillus cereus* for 24-48 h during ergo fermentation (Buyser*et al.,* 2001). Since ergo is commonly consumed soon after 24 h of fermentation, the survival of pathogen beyond 24 h would be undesirable and threat for consumers health (Tsegaye and Ashenafi, 2005).

2.4.3. *Ititu*

The name *ititu* is used for concentrated fermented milk prepared and consumed by the Borana tribes in southern Ethiopia. This pastoral/farmer community prepares ititu during the rainy season when milk is available in abundance for later consumption during the drier seasons when fresh milk supply is markedly scanty (Almaz *et al.,* 2001). The product has good keeping quality and remains acceptable for about two months at ambient temperature ($25^{O}C$-$30^{O}C$) and can be stored from about two months (Kassaye *et al*., 1991). *Ititu* is consumed as side dish with traditional porridge or thin-baked cereal chips. It can also be consumed as food or drink alone. It is considered as one of the special foods and served to much respected guests as well as to weaning-age children and the elderly.

The traditional production way of ititu is by collecting fresh milk in a well-smoked fermenting vessel called *gorfa* (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* can be ready for use after washed with hot water, air dried, rinsed with fresh milk and smoked for a few minutes with splinters of *Acacia nilotica* or other plants. It also can be treated with leaves of *Ocimumbasilicum* for cleaning and imparting desirable flavor to the product. *Ititu* has more contents of free and total amino acids when compared to fresh whole milk and is rich in amino acids such as glutamic acid, alanine, proline, leucine and serine (Kassaye *et al*., 1991).

Studies showed that *ititu* has 3.3 - 3.7% fat, 3.3 - 3.6% protein and 3.3 - 3.5% lactose (Fekadu1997). Total bacterial count was reported to be 10^{12} cfu/g, mainly dominated by lactic acid bacteria (Kassaye *et al*., 1991). The prevalent lactic acid bacteria in *ititu* are *Lactobacillus*

casei and/or *Lactobacillus plantarum* with lactic acid bacteria count of 10^8 cfu/g (Almaz *et al*., 2001).

2.5. Nutritive and Structural Characteristics

2.5.1. Probiotics

In addition to yoghurt culture, probiotic application of starter cultures in fermented dairy products manufacture is becoming more and more interesting because of their therapeutic and prophylactic characteristics. These bacteria beneficially affect human health by improving the balance of intestinal micro flora. The two most important genera in the probiotic field are *Lactobacillus* and *Bifidobacterium*, but some others contain species of interest, e.g. *Pediococcus, Enterococcus* and *Lactococcus*. For selection, preferably the microbes should have GRAS (Generally Regarded as Safe) status, a long history of safe use in foods, be non-pathogenic, and acid and bile tolerant (Doder *et al.,* 2013).

To successfully develop fermented dairy products containing probiotics, it is important to understand the growth characteristics of probiotics in order to optimize their survival during processing conditions. One of the major limitations of the incorporation of *Bifidobacteria* is the pH value of product and the aerobic conditions of production (Boylston, 2004). In practical application, most strains of *Bifidobacteria* are sensitive to pH values below 4.6. Because of that, the pH value in the final product must be maintained above 4.6. Therefore, fermented milks are not optimal for the maintenance of high concentration of some strains. Certain approved strains of LAB. Acidophilus and *bifidobacteria* are the most widely used bacteria during the manufacture of probiotic yoghurts (Shikha, 2016).

2.5.2. Exopolysaccharides

Incorporation of probiotic bacteria with ropy or non-ropy starter culture in fermented dairy products has been studied over the last years by many researchers (Mende*et al.,* 2013 and Prasanna*et al.,* 2013). Ropy cultures are capable of producing extracellular polysaccharides (EPS) during the fermentation process, in contrast to non-ropy cultures (Tamime, 2006). Streptococci, lactobacilli and *lactococci* are some EPS-producing bacteria species which have been successfully used to produce yoghurt with improved physicochemical properties (Prasanna *et al.,* 2013). Exopolysacharides (EPS) can improve technological characteristics of fermented dairy products, like stability and texture, and may also offer protection to cell against phage attack, desiccation and osmotic stress, thus behaving as prebiotics and improve immunity to fight against pathogenic organisms (Mende et *al.,* 2013).

2.5.3. Prebiotics

Inulin and lactulose are prebiotic which are used as non-digestible ingredients beneficially affect humans since they selectively stimulate growth and/or activity of one of limited number bacteria in the colon (Debon *et al.,* 2010). Inulin is a soluble and fermentable fiber named fructan with 2–60 fructose units linked via β (1–2) bond. It is partly soluble in water (10 %, 20 °C) and has a sweetening power of 15 % of sucrose. The extensive use in dairy industry is based on nutritional and technological properties of inulin (Meyer *et al.,* 2011).These include improved bowel habits (Marteau *et al.,* 2011), increased calcium absorption with positive effects for bone health, a lowering of serum lipids with relevance for heart health (Brighenti, 2007), a positive effect on feeling of satiety with potential positive consequences for weight management, a potential effect to enhance resistance to infections and to stimulate the immune system (Cani *et al.,* 2006). Inulin

at high intakes (20 g/day) might also decrease plasma triglycerides and cholesterol and might improve glucose tolerance in diabetic (Shikha, 2016).

The technological use of inulin is based on its properties as a sugar replacer (especially in combination with high intensity sweeteners), fat replacer and texture modifier. For fat replacement in low-fat dairy products inulin seems particularly suitable as it may contribute to an improved mouth feel (Piccinah and Eberhard, 2009). Several principal health effects are studied so far and could be divided into two groups. The first group refers to "nutritional function", which means function of supplying more nutrition efficiently. The second group is "physiological function", which includes the therapeutic functions beyond nutritional effects (Cifelli *et al*, 2011).

Bioactive components are constituents in food or dietary supplements that meet basic nutritional needs. They are responsible for health changes and are capable of eliciting a biological response, such as reducing hypertension, stimulation of the immune system preventing cancer, enhancing lean body mass, or killing bacteria (Siro *et al.*, 2008). Nowadays, bioactive peptides have gained increasing attention for human health promotion and deferment of age related diseases (Korhonen, 2009 and Urista *et al.*, 2011).

2.6. Lactic Acid Bacteria (LAB)

Lactic acid bacteria (LAB) comprise a diverse group of bacteria which are Gram positive, non-spore forming cocci, cocco-bacili or rods. In most cases they are anaerobic, Microaerophilic or aerotolerant in their oxygen demands. Although some may produce pseudocatalase when grown at low sugar concentrations, they are generally catalase and oxidase negative (Desalegn, 2013). Lactic acid bacteria represent as the most extensively studied microorganisms for milk fermentation (Maragkoudakis *et al.*, 2006). The presence of LAB in milk fermentation can be

either as spontaneous or inoculated starter cultures. Milk itself is known as one of the natural habitats of LAB (Delavenne *et al.*, 2012). Although under spontaneous fermentations the growth of LAB cannot be predicted or controlled, but this procedure has been practiced and carried out traditionally for years (Yantyati, 2014).

Milk fermentation process has been relied on the activity of LAB, which play a crucial role in converting milk as raw material to fermented milk products. In milk fermentation industry, various industrial strains of LAB are used as starter cultures (Mayra and Bigret, 2004). Starter cultures of LAB were obtained from sequence activities and passed a process of isolation, selection and confirmation. Several behaviors as the characteristics of each individual selected strains of LAB has been established and used in the production of fermented milk products industrially. The most important properties of LAB are their ability to acidify milk and to generate flavor and texture, by converting milk protein due to their proteolytic activities (Yantyati, 2014).

Lactic acid bacteria are found in two distinct phyla, namely *Firmicutes* and *Actinobacteria.* Within the *Firmicutes* phylum, the most important genera of LAB are *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus* and *Weissella*, which all belong to the order *Lactobacillales* and are low-GC content organisms 31–49 % (Horvath *et al.*, 2009). Within the *Actinobacteria* phylum, LAB belongs to the *Bifidobacterium* genus, which has high-GC content 58–61 % (Klaenhammer, 2008).

Despite the functional definition characterizing members of the LAB, they are very different from a taxonomic viewpoint (Salvetti *et al*., 2013). Based on the phylogenetic relatedness of 16S ribosomal ribonucleic acid (16S rRNA) sequences from different species, LAB have been divided into two major branches, the *Clostridium* branch and the *actinomycetes* branch. The

typical LAB, such as *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus, Enterococcus* and *Pediococcus,* belong to the *Clostridium* branch. In contrast, the genus *Bifidobacterium* belongs to the *actinomycetes* branch (Stiles and Holzapfel, 1997).

2.6.1. The Genus *Bifidobacterium*

Bifidobacterium species are non-motile, non-spore forming, non-gas producing, Gram-positive, catalase-negative bacteria with a high GC content (46–67 %)(Biavati and Mattarelli, 2006).Their morphology is generally referred to as bifid or irregular V- or Y-shaped rods resembling branches. The actual reason for the irregular shape of *bifidobacteria* is not yet clearly understood. Majority of species are obligate anaerobes (Okamoto *et al*., 2008). The optimum growth temperature is 37–41^{0}C for most species with minimum and maximum growth temperatures of 25 and 45^{0}C, respectively. The optimum pH for initial growth is 6.5–7.0 (Biavati and Mattarelli, 2006).

Traditionally, species of *Bifidobacterium* have been identified on the basis of the location or host from which they were isolated, example for, *B. animalis*, *B.adolescentis* and *B. dentium*, their cell morphology, from analysis of fermentation products and associated enzyme activities, or by their ability to utilize various sugar substrates (Ventura *et al*., 2004a).

In milk, *bifidobacteria* can only be propagated if "bifidofactors" such as lactulose, N-acetyl glucosamine, and peptide hydrolysates are added (Michael, 2008).

2.6.2. The Genus *Enterococcus*

The genus *Enterococcus* belongs to the family *Enterococcaceae,* order *Lactobacillales,* phylum *Firmicutes* and is the type genus of *Enterococcaceae* family (Pavel *et al*., 1984). More than 200 genomes from isolates of *Enterococcus* species have been sequenced and are available including

the sequences of *E. casseliflavus, E. cecorum, E. columbae, E. durans, E. faecalis, E. faecium, E. gallinarum, E. hirae, E. italicus, E. mundtii*and *E. saccharolyticus* (Devriese *et al*., 2006).

No phenotypic criteria are available by which the genus *Enterococcus* can be separated clearly from other genera. However, species within the genus have common attributes. The cells are ovoid, Gram-positive and occur singly, in pairs or in short chains. Within the chains the cells are frequently arranged in pairs and elongated in the direction of the chain. The classical species *E. faecalis* and *E. faecium* on which the genus description was based, have a number of characteristics in common that distinguish them from other catalase negative, Gram-positive, facultative anaerobic cocci with which they could be confused: their ability to grow both at 10 m and 45 ^{0}C, in 6.5 % NaCl broth and at pH 9.6 (Rahkila *et al*., 2011). However, other species, added later to the genus, do not show all of these characteristics. In cases where only presence or absence of the genus *Enterococcus* in a sample is required, it is only possible to determine by identification of the component species (Naser *et al*., 2005).This means that genus identification necessarily follows species identification: e.g. when an isolate shows characteristics of one of the species described below, the isolate is an *Enterococcus.* For practical reasons if an isolate has the following attributes it can be presumed to belong to the genus *Enterococcus*: catalase negative, Gram-positive cocci, able to grow in 6.5 % (w/v) NaCl broth, showing good growth on media containing 0.04 % sodium azide (commonly used in selective isolation of *Enterococcus* species (Martin *et al*., 2005).

2.6.3. The Genus *Lactobacillus*

The genus *Lactobacillus* consists of Gram-positive, non-spore-forming rods that are usually non motile and occasionally nitrate reducers (Hammes and Hertel, 2006). They are catalase negative

when growing in complex nutritional environments, such as carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives and vitamins. *Lactobacillus* species generally utilize glucose by fermentative via the Embden-Meyerhof-Parnas pathway or glycolysis, and may be either homo-fermentative, producing more than85 % lactic acid from glucose, or hetero-fermentative, producing lactic acid, CO_2, ethanol and/or acetic acid in equimolar amounts (Douillard *et al*., 2013).

The genus *Lactobacillus* is the largest, most diverse group among the LAB, and currently comprised 158 validated species. While *Lactobacillus* can be found in diverse habitats, they can be characterized within eight niche types according to the isolation source: plant (isolated from plant or plant-associated fermentation products), sourdough, meat products, dairy products, wine products, animal GI (isolated from human or animal gastrointestinal tracts, including strains from saliva and feces), animal-NGI (other human and animal-derived isolates with the exclusion of GI isolates) and the environment in general (Hammes and Hertel, 2009).

Lactobacillus species are generally anaerobic, but can be aero-tolerant, and aciduric or acidophilic (Zhang *et al*., 2011). Cells vary from long and slender, sometimes bent rods, to short, often coryne form, cocci; chain formation is common in rod-shaped forms and these chains are usually regular in width and length (0.5 -1.2 9 1.0−10.0 μm). For growth, the temperature range is between 2^0c and 53 ^{0}C, and the pH range is between 3 and 8. Optimal growth temperature and pH are usually 30^0c –40 ^{0}C and 5.5–6.2, respectively (Salvetti *et al*., 2012).

2.6.4. The Genus *Lactococcus*

Lactococci are coccoid Gram-positive, catalase-negative, non-motile and facultative anaerobic bacteria, with L-(+)-lactic acid as their predominant end product ofglucose fermentation. They are commonly called mesophilic lactic *streptococci*and most strains react with group N antisera

(Tanigawa and Watanabe, 2011). *Lactococci* havecomplex and variable nutritional requirements, and they typically inhabit plants, animals and their products (Xu *et al*., 2013).The common morphology of *lactococci* consists of 0.5- to 1-μm diameter spheres or ovoid cells that exist in pairs or chains. Cells of *lactococci* often elongate in the direction of the chain, which makes them difficult to differentiate from *lactobacilli* (Passerini *et al*., 2010).

Lactococcal cultures usually grow in the range of 10–40^{O}C, although some species may grow under temperatures as low as 7^{O}C upon prolonged incubation for 10–14 days (Sakala *et al*., 2002). Cultures typically grow in 4.0 % (w/v) NaCl; however, *Lac. lactis subsp. Cremoris* tolerates only 2.0 % NaCl, which is the only known exception. *Lactococci* grow best at near-neutral pH values in media but cease to grow at about pH 4.5. They are homo-fermentative microaerophilic bacteria, and their growth is not severely affected by aeration. However, highly toxic oxygen compounds such as superoxide, hydrogen peroxide and hydroxyl radicals are generated by *Lac.lactis* under aerobic conditions. *Lactococci* are catalase negative, but they have NADH oxidases and superoxide dismutases, which are unregulated under aerobic stress conditions (Wegmann *et al*., 2007).

The phylogenetic position of *lactococci* within Firmicutes was established by comparison of the 16S rRNA gene sequences along with *Streptococcus* and *Lactovum*, the *Lactococcus* genus is a member of the family *Streptococaceae*, in the order *Lactobacillales*. Therefore, a close phylogenetic relationship exists among the genera *Lactococcus*, *Streptococcus* and *Enterococcus* (Perez *et al*., 2011). At present, the genus *Lactococcus* comprises 11 recognized species and subspecies. *Lactococcus lactis* is considered the most important industrial dairy starter microorganism and has been used for hundreds of years (Kutahya *et al*., 2011).

2.6.5. Genus *Leuconostoc*

Members of genus *Leuconostoc* are Gram-stain positive, non-motile, non-spore forming, facultative anaerobic, ellipsoidal to spherical cells, often elongated, and arranged in pairs or chains (Tanigawa and Watanabe, 2011). *Leuconostoc spps.* are environmental organisms often found in association with plant material, milk, dairy products, meat and other food products. These species are similar to hetero-fermentative *Lactobacilli*, especially gas-forming *Lactobacillus* confuses and *Lactobacillus* viridescens. *Leuconostoc* and *Lactobacillus* are often reported to be isolated from the same habitat and share many characteristics (Lee *et al*., 2012). *Leuconostoc spp*. is intermediate forms between *streptococci* and *Lactobacilli* (Sabat *et al*., 2013).Genus *Leuconostoc* comprises the following 13 recognized species: *Leuc. mesenteroides, Leuc. carnosum, Leuc. citreum, Leuc. fallax, Leuc. gasicomitatum, Leuc. gelidum, Leuc. inhae, Leuc. kimchii, Leuc. lactis, Leuc. holzapfelii, Leuc. pseudomesenteroides, Leuc. palmae and Leuc. miyukkimchii* (Kim *et al*., 2003).

Currently, 13 *Leuconostocs spps* have been identified. Typing methods for identifying different types of organisms within species are essential, epidemiological tools in infection prevention and control (Sabat *et al*., 2013). Typing methods are divided into two major categories, i.e. phenotypic and genotypic methods. Traditional phenotyping methods, such as serotype, biotype, phage-type or anti-biogram, have been used for many years to isolate and characterize *Leuconostoc* and sometimes, to identify between species and sub-species. Compared with the phenotypic-typing methods, genotypic-typing methods also have some advantages, such as general applicability and high discriminatory power. Currently, several molecular-typing approaches have been attempted to analyze *Leuconostoc spp*. (Alegrı *et al*., 2013).

2.6.6. The Genus *Pediococcus*

The genus *Pediococcus* is typical of lactic acid bacteria (LAB) in being Gram positive, catalase negative and oxidase negative. Species in this genus grow under facultative aerobicto microaerophilic conditions. They are homo-fermentative and produce lactic acid but not $CO_2$from glucose and are unable to reduce nitrate (Holzapfel *et al*., 2009). They are the only LAB that divides alternately in two perpendicular directions to form tetrads, which thus *pediococci* often differ from all other LAB. They never form chains typical of the other genera of *coccoid* LAB such as *Leuconostoc, Lactococcus* and *Streptococcus* species, which also only form chains as a result of division in one plane (Martino *et al*., 2013).Currently, the genus *Pediococcus comprises 11 validly described species, P. acidilactici, P. argentinicus, P. cellicola, P. claussenii, P. damnosus, P. ethanolidurans, P. inopinatus, P. parvulus, P. pentosaceus, P. siamensis*and *P. stilesii* (Midha *et al*., 2012).

Pediococcus species have coccoid cells that divide in two planes at right angles to each other forming tetrads, but never chains (Holzapfel *et al*., 2009). The cells may occur singly or in pairs, especially during the early or mid-logarithmic growth phase. The coccoid cells are perfectly spherical and rarely ovoid, ranging in diameter from 0.6 to 1.0 μm. *Pediococcus* species are non-motile, do not form spores, are not capsulated and are characterized as Lys-D-Asp peptidoglycan types (Pittet *et al*., 2012).

As typically homo-fermentative bacteria, *Pediococcus* species ferment carbohydrates via the Embden-Meyerhof parnas (EMP) pathway. With the exception of *P. argentinicus* and *P. claussenii* that form L (+) lactate from glucose, the other species all form DL (+) lactate. *Pediococcus* species are generally tolerant to high salt concentrations (growing at [18 % NaCl [w/v]) and can grow at pH values as high as 9.0 but not at pH 5.0 (Haakensen *et al*., 2009).

Characteristics generally used for species identification include the range of temperatures, pH and NaCl concentration at which growth occurs, and physiological characteristics such as how they ferment different carbohydrates, hydrolysis of arginine and which isomers of lactic acid are produced (Holzapfel *et al*., 2009). However, it is impossible to unequivocally identify *Pediococcus* species using these classical phenotypic methods. To accurately identify different molecular and biological approaches have been developed (Calmin *et al*., 2008).

2.6.7. The Genus *Streptococcus*

The genus *Streptococcus* consists of Gram-positive, non-motile, spherical or ovoid cells that are typically arranged in pairs or chains when grown in liquid media. All species are facultative anaerobic, some requiring additional CO_2 for growth. They are non-spore forming, catalase negative, homo-fermentative and have complex and variable nutritional requirements (Jensen et al., 2012). They metabolize carbohydrates by fermentation resulting mainly in lactic acid but no gas. Their temperature optima are usually around 37^oC, but maximum and minimum temperatures vary somewhat among species. Many species are pathogenic to man and animals and some are highly virulent (Boers *et al.*, 2012).

Some species, e.g. *S. mutans*, grow as short rods under some cultural conditions and several of the other oral species appear to be pleomorphic at the time of primary isolation. Individual cells are usually 0.8–1.2 μm in diameter but chain lengths vary from a few cells to over 50 depending on the species, isolate and culture conditions (Maeda *et al*., 2011).

Some of them produce capsules, either of hyaluronic acid as in *S. pyogenes*, or of a variety of type-specific polysaccharides as in *S. pneumonia* but this is not a consistent characteristic throughout the whole genus. Several species produced extracellular polysaccharides when grown in the presence of sucrose, including both glucans and fructans (Milinovich *et al*., 2008). Growth

on solid media is often enhanced by the addition of blood, serum, or glucose. For optimal growth in liquid media, the addition of glucose or some other fermentable carbohydrate is essential, but the rapid fall in pH quickly inhibits growth unless the medium is strongly buffered, as it is in or the pH is controlled by the continuous addition of an alkali (Takada *et al.*, 2013).The genus *Streptococcus* is a member (type genus) of the family *Streptococaceae* and it falls within Gram-positive eubacteri of Clostridium or Bacillus branch which contains the low (<50 mol. %) G+C. Following extensive taxonomic revision of Streptococcus and discovery of new species the genus currently consists of over 100 species and subspecies (Zhang *et al.*, 2013).

2.7. Starter cultures

Starter cultures are used in the manufacturing of different types of fermented milk and dairy products including yogurt, dahi, cultured buttermilk, sour cream, quarg, kefir, koumiss, and cheese. The use of these cultures for the preparation of products has been practiced since time immemorial (Delzenne *et al.*, 2011).Traditionally, the common method was to use the previous-day's product (i.e., milk, dahi, whey, butter milk, etc.) as an inoculum to produce the fresh batches of fermented product. Such methods were not reliable and often resulted in off-flavor, inconsistent products, and product failure due to undesirable fermentation. These drawbacks were mainly due to the lack of scientific knowledge of starter culture technology. The ideal of pure culture for making good-quality fermented milk became more visible in the mid-nineteenth century, where starters were widely studied and their metabolisms were well established (Bettache *et al.*, 2012).

This leads to manufacturers handling large volumes of milk started selection of appropriate starters to obtain uniform quality product. This made the selection of appropriate starters even for manufacturers' handling large volumes of milk to obtain uniform quality of product.

Thereafter, companies started producing pure cultures for commercial application (Padalino *et al.,* 2012).

Starter cultures may be defined as the carefully selected group of microorganisms that are deliberately added to milk and milk products to bring desirable fermentative changes. These have multi-functional role in dairy fermentation; the primary one is to produce lactic acid, hence, popularly called as lactic acid bacteria (LAB). Besides, production of lactic acid, certain cultures perform secondary functions such as the production of acetic, propionic, and folic acids, CO_2, H_2O_2, ethanol, bacteriocins, exopolysaccharides (EPS), and soon (Yang *et al*., 2012).

2.7.1. Functions of Starter Cultures

During manufacturing of fermented food products starter cultures have various functions. (Behare *et al.,* 2009), thus are:-

- Produce lactic acid and other metabolites (i.e., alcohol, CO2, propionic acid, acetic acid, etc.).
- Produce aromatic compounds like diacetyl, acetaldehyde, andacetoin.
- Control the growth of pathogens and spoilage causing microorganisms.
- Produce certain vitamins (i.e., folic acid, vitamin B12, niacin,etc.).
- Bring proteolytic and lipolytic activities.
- Improve body and texture of certain products by producing EPS.
- Assist in overall acceptability of the final product.

2.7.2. Preservative Property of Lactic Acid Bacteria

The most well-known characteristics of LAB related to preservative property are their ability to produce acid, which in turn exhibit antimicrobial activity. Acidification of the milk protects the milk against spoilage microorganisms and proliferation of pathogens. LAB also releases

antimicrobial metabolites so called bacteriocins (Zacharof and Lovitt, 2012). Both acids and bacteriocins are great potential to be used in food preservation, which are considered as safe natural preservatives (Yantyati, 2014).

2.7.3. Acid Production

Organic acids are the end product of carbohydrate metabolism produced by LAB. Homo fermentative species of LAB convert sugars in milk mostly into lactic acid, whereas the hetero fermentative species convert lactose into lactic acid, acetic acid, ethanol and CO_2. Production of lactic acid by LAB is strain depended (Yantyati, 2014).

2.7.4. Bacteriocins Production

Bacteriocins are substances of protein structure, either proteins or polypeptides, that possessing antimicrobial activities and produced during the primary phase of bacterial growth. Generally bacteriocins only active against closely related bacterial species (Yantyati, 2014).Among the bacteriocins produced by LAB, nisin produced by *Lactococcuslactis*spp., is the only bacteriocin that has been officially employed in the food industry and its use has been approved worldwide (Zacharof & Lovitt, 2012). Bacteriocins produced by wild *L. lactis*strains isolated from traditional starter free-cheese made from raw milk were reported as nisin A, nisin Z and lactoccocin 972 (Alegría & López, 2010). Milk fermented with *L. lactis*W8 can be used as a rich source of nisin for commercial purposes. Pediocin PA-1 produced by *Pediococcusacidilactici* is an equally promising bio preservative in foods as nisin (Mitra & Biswas, 2010).

2.7.5. Flavor compound production

The most common LAB cultures used in yoghurt manufacture is *Streptococcus thermophilus* and *Lactobacillusbulgaricus.* They, in association and synergistically, produce volatile metabolites that determine the flavor of yoghurt. The mutual benefit between them occurred by releasing the

amino acids from the milk as well as organic acids and therefore they produced more lactic acid and aromatic compounds. Flavor of yoghurt are supported by various compounds, in which lactic acid represents as the major contributor, and other aroma compounds (Routray & Mishra, 2011)

2.8. Important pathogenic microorganisms present in raw milk

Raw milk can be a source of food-borne human diseases caused by pathogens. Their prevalence, like other non-pathogenic microorganisms, is affected by numerous factors such as farm size, number of animals, hygiene, farm management practices, geographical location and season (Oliver *et al.*, 2005). Pathogenic microorganisms can be transferred to raw milk either from animals, i.e., zoonotic pathogens or from contaminated environment, i.e., exogenous pathogens. Most of the pathogenic microorganisms in milk can cause the three types of microbial food-borne diseases:

A. milk-borne infection,
B. milk-borne intoxication and
C. Milk-borne toxico-infection (Ray, 2004).

Table 1: Overview of significant pathogenic microorganisms present in raw milk

Organism	Disease	Main source of contamination
Entero-bacteriaceae		
Escherichia coli, including 0157:H7	Gastroenteritis, hemolytic uremic syndrome, thrombotic throm-bocytogenicpurpua (TTP)	Feces, water, Bio films on the milking equipment
Salmonella	*Gastroenteritis, typhoid fever*	*Feces, water*
Shigella	*Gastroenteritis*	*Feces, water*
Yersinia enterocoliticaa	*Gastroenteritis*	*Feces, water*
OtherGram-negative bacteria		
*Aeromonashydrophila*a	Gastroenteritis	Cold-stored milk
*Brucella*spp.	Brucellosis (Bang's disease)	Sick animal
Campylobacter jejuni	Gastroenteritis	Feces
*Pseudomonas aeruginosa*a	Gastroenteritis	Cold-stored milk
Gram-positive spore-formers		
*Bacillus cereus*b	Intestinal intoxication	Soil, bio films
Bacillus anthracis	Anthrax	Soil
Clostridium perfringens	Gastroenteritis	Silage feed, air
Clostridium botulinum	Botulism	Silage feed, air
Gram-positive cocci		
Staphylococcus aureus	Emetic intoxication	Mastitis udder
Streptococcus agalactiae	Sore throat	
Streptococcus pyogenes	Scarlet fever, sore throat	
Streptococcus zooepidemicus	Pharyngitis, nephritic sequelae	

(Oliver *et al.* 2009).

2.8.1. Escherichia coli

Escherichia coli belong to the family of *Enterobacteriaceae* and have been recognized as the most important indicator of fecal contamination of water and raw food products. *E. coli* strains are responsible for three main clinical syndromes, namely the enteric and diarrheal diseases, urinary tract infections and sepsis/meningitis, and thus they have grouped into entero-aggregative *E.Coli* (EAggEC), entero-invasive *E.coli* (EIEC), entero-pathogenic *E.coli* (EPEC) and entero-toxigenic *E.coli* (ETEC) (van Kessel *et al*., 2011).

Strains of the latter group produce cytotoxins called verotoxins (VTEC) or shigatoxins (STEC) and colonize in the intestinal track of healthy animals. *E. coli* serotype O157:H7 (EHEC) is the most studied strain of *E. coli*, followed by $_{O26}$ and O111 serotypes belong to STEC group (also called enterohemorrhagic *E. coli*) (Jooste & Anelich, 2008). Most of *E. coli* strains are not heat-resistant and are readily destroyed by the pasteurization process; however, EHEC is an acid-resistant strain and thus can grow on acidified milk products such as yogurt and fresh acid cheese (Lekkas *et al.*, 2006). Cattle feces are considered as a major reservoir of EHEC (Weimer, 2001)

2.8.2. *Staphylococcus spp*

Staphylococcus aureus belongs to the family of *Micrococcaceae* and is the most significant causative agent of mastitis in dairy cows. The enterotoxigenic strains produce enterotoxins that are classified according to serotypes into A-H groups and toxic shock syndrome toxin (TSST) (Oliver *et al.,* 2005). Staphylococcal enterotoxin is heat-resistant and survives pasteurization (Oliver *et al.,* 2009). Prevalence of *S. aureus*in raw bovine and caprine milk samples from Norwegian dairies was reported at rates from 47 to 75% and from 2 to 96%, respectively (Jakobsen *et al.,* 2011). Coagulase-negative *Staphylococcus* spp. was isolated at levels of >74% and the dominant species reported were *S. chromogenes*, *S. hyicus*and, *S. epidermis* (Oliver *et*

al., 2009). *Staphylococus aureus* outbreaks were reported to be more related to the consumption of raw milk cheeses than the consumption of raw milk (Rosengren *et al.,* 2010).

2.8.3. *Salmonella*

The genus *salmonella* belongs to the family *enterobacteriaceae*. As the name indicates *enterobacteriaceae*is comprised of bacteria that proliferate in the intestine. Several genera of this family include pathogenic species. Like other *enterobacteriaceae*genera, *salmonella* is a gram negative flagellated road shaped bacterium. They are facultative anaerobic with both respiratory and fermentative metabolic pathways (Kuhn *et al*., 2011).

Salmonella is found in the environment and the gastrointestinal tract of wild and farmed animals. Animals may become infected with *Salmonella* through environmental contamination, other animals or contaminated feed. Both animals and humans can function as *Salmonella* reservoirs. In addition to sheep, goats, cattle, chickens and pigs, other animals which can become infected with *Salmonella* include geese and other birds, lizards and other reptiles, shellfish, and amphibians such as turtles. Indeed, most *Salmonella* contamination is of animal origin (Freitas *et al.,* 2010).

Salmonella spp. is the most common pathogenic bacteria associated with a variety of foods. Although myriad foods can serve as *Salmonella* sources, meat and meat products, poultry and poultry products, and dairy products are significant sources of food-borne pathogen infections in humans. Presence of *Salmonella spp*. in fresh raw products can vary widely (Castro *et al.,* 2010). Frequency usually ranges from 1 to 10 %, depending on a range of factors including organism, farming and/or food production practices, and geographical factors (Harris *et al.,* 2003).

Salmonella serotypes can grow and survive on a large number of foods (Harris *et al.*, 2003). Their behavior in foods is controlled by a variety of environmental and ecological factors, including water activity, pH, Eh, chemical composition, the presence of natural or added antimicrobial compounds and storage temperature; as well as processing factors such as heat application and physical handling. For example, optimum pH for growth in Salmonella is approximately neutral, with values > 9.0 and < 4.0 being bactericidal. Minimum growth in some serotypes can occur at pH 4.05 (with HCl and citric acids), although this minimum can occur at pH as high as 5.5, depending on the acid used to lower pH (Harris, *et al.*, 2003). Growth in *Salmonella* can continue at temperatures as low as 5.3 °C (*S. Heidelberg*) and 6.2 °C (*S. Typhimurium*), and temperatures near 45 °C (temperatures ≥ 45°C care bactericidal). In addition, available moisture (aw) inhibits growth at values below 0.94 in neutral pH media, although higher aw values are required as pH declines to near the minimum growth values (Secretaria, 2011).

2.8.4. *Listeria monocytogenus*

Listeria monocytogenus is a gram-positive, facultative intracellular rod bacterium that is catalase positive and beta-hemolytic when grown on blood agar. There have been several historical food-borne illness breakouts involving *L.monocytogenus*. In 1981, *L. monocytogenus* was revealed to be a food-borne illness linked to a variety of foods. While *L. monocytogenus* is not the most common food-borne illness, it has the highest mortality rate secondary to its unique virulence factors (Jackson *et al.*, 2018).

L. monocytogenus' virulence factors include but are not limited to intracellular mobility via actin polymerization and the ability to replicate at refrigerator temperatures. This makes it difficult for food industries to control. Transmission of the bacteria occurs via the fecal-oral route and most

commonly involves foods such as cold deli meats and unpasteurized dairy products. The number of cases involving *L. monocytogenus* has decreased in recent years thanks to advances in prevention, detection, and treatment (Jordan, 2018).

Infection via *L. monocytogenus* (listeriosis) includes but is not limited to sepsis, meningitis, encephalitis, spontaneous abortion, or fever and self-limiting gastroenteritis in a healthy adult. Populations at the most risk for *L. monocytogenus* infection include pregnant females, infants, immuno-compromised individuals, and elderly (Ranjbar *et al.,* 2018).

Listeria commonly causes meningitis in the young (neonates), elderly, and immune compromised patient population. Healthy individuals infected with L. monocytogenus typically have a self-limiting gastrointestinal infection with fever and diarrhea. The *Listeria* family consists of 10 different species with L. monocytogenus found most consistently in humans. L. monocytogenus has 13 different serotypes based on a variety of flagella and surface antigens. However, there are only three serotypes that inflict disease in humans (Komora *et al*., 2018). L. monocytogenus is ubiquitous as it can be found in soil, water, and decaying vegetation. The bacterium can also be found in the human digestive tract. Foods that have the highest rates of L. monocytogenus related infections include: raw sprouts, unpasteurized milk, soft cheeses, cold deli meats, cold hot dogs and smoked sea food (Hunt *et al*., 2018).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

This study was carried out in Sodo zuria District, Wollaita zone, Southern Ethiopia. The area is located about 390 km south of Addis Ababa between 6º4´N to7º1´N and 37º4´E to 38º2´E. The area has an altitude range of 700-2950m above sea level and receives rainfall between 450-1446 mm (WZFEO, 2015).

3.2. Study Design, Sampling Technique and Methods of Data Collection

3.2.1. Survey Study

A cross sectional study design was used to collect data during February through April 2020. The three *kebeles* were purposively selected from Sodozuria district based on milk production potential and agro ecology difference. These are *Kokatemarachere* (highland), *Bossa kecha* (midland) and *Gulgula* (lowland). Multistage sampling method was used. Based on this, a total of 757 households having two and more milking cows were identified as total population in the three *kebeles*. The households were stratified based on agro ecology. Accordingly 368 from *Kokatemarachere* (highland), 212 from *Bossa kecha* (midland) and 177 from *Gulgula* (lowland) were identified as total population representing the sampling frame. Then a total of 162 households were randomly selected from the sampling frame as representative sample. The number of samples from each agro ecology, was determined by using quota sampling method. Thus, 48.61% (79 households) from *Kokatemarachere*, 28% (45 households from *Bossa kecha* and 23.38% (38 housholds) from *Gulgula* were selected. Primary data was obtained by direct interviewing of farmers through the modified and semi-structured questionnaire.

3.2.2. *Shimpia* Sample Collection

A total of 30 *Shimpia* samples (10 from each kebele) were taken with a 300ml sterile bottle immediately after prepared in the farmer's house. Three samples from each kebele, once a week in the morning were collected simultaneously. Then it was brought to Hawassa University dairy science laboratory and kept in a refrigerator (4°C) until analysis. All the analyses were done in triplicate and the results reported as mean ± standard deviation.

3.2.3. Physicochemical Analysis of *Shimpia*

Total solids % (w/w), ash % (w/w), total proteins % (w/w), total fat % (w/w), and pH of *Shimpia* samples were determined according to the Official Methods of Analysis (AOAC, 2005) as follows.

Determination of pH

The pH of *Shimpia* samples was measured using a digital pH-meter (Accument pH-meter, Model number 910, Fisher Scientific). Before use, the pH-meter was calibrated using standard buffer solutions of pH 4.0 and pH 7.0.

Total solid (TS) Content Determination

The total solids content of the *Shimpia* was determined by oven drying at 105°C overnight (12 hours). Moisture and the total solid content were determined according to AOAC (2005) methods. Briefly, 5 g of sample was weighed into a dry and pre-weighed crucible and the samples were labeled carefully using a pencil. The crucible and its content then was transferred into the oven at a temperature of 105°C and dried for 12 h. The crucibles were allowed to cool in desiccators and weighed. The crucible was returned into the oven for another half hour and again cooled and reweighed. The process was repeated until a constant weight was reached. Total solids content was calculated according to the following formula:

Total solids (%) = $\frac{Weight\ of\ dried\ sample}{Weight\ of\ original\ sample} x100$

Ash content determination

The ash content of *Shimpia* was determined by incinerating 1 g of previously dried sample during dry mater determination in a muffle furnace at 550°C for 6 h. After cooling in desiccators for a period of 45 minutes, the% of total ash content was calculated on a dry basis according to Official Methods of Analysis (AOAC, 2005).

Protein content

The total protein content of *Shimpia* was determined by the Kjeldahl method (IDF standards 20B, 1993) using modified amounts of sample and reagents. Samples of 1gm *Shimpia*, 3ml of concentrated sulfuric acid and one kjel tablets containing 1.5gm KSO_4 and 7.5mg selenium were added. After complete digestion and prior to distillation 30ml distilled water and 25ml NaOH (33w/v%) were added to 4% boric acid, and the distillate was titrated against 0.01N HCL 4% boric acid as an indicator. The protein content of samples was calculated from the nitrogen content using a conversion factor of 6.38.

Fat content

The fat content of *Shimpia* was determined according to the Official Methods of Analysis (AOAC, 2005). Thus 10 ml sulphuric acid was added to the butyrometer (8 calibration) followed by 11 ml of well-mixed sample. Then 1 ml of Amyl alcohol was added and immediately stopper was inserted and butyrometer was shook gently until the curd was dissolved and no white particles were seen. The butyrometer was kept in the water bath at 65ºC until a set was ready for centrifuging. Finally, the butyrometer placed in the centrifuge for 5 minutes at 8,000 rpm and kept in the water bath at 65ºC for other 5 minutes to be settled and ready for reading.

3.2.4. Microbial Analysis of *Shimpia*

The microbial analysis was done by following the following method described by Ahemed and Carolyn (2003). To prepare serial dilution, 7 test tubes each having 9 ml sterile peptone water was prepared. Then 1 ml of well-mixed sample was aseptically transferred to the first test tube. Then by transferring 1ml dilute to the next test tubes serial dilution was prepared up to 10^{-7} dilution. The pour plate method has been used to culture microbes. One ml of sample from each dilution was poured into pre-sterilized and dried plates.Then15-20ml of sterilized media with temperature of 42^0c was poured over each sample in the plate and was mixed gently for even distribution of sample.

Total *coliform* bacteria count was determined using Violet red bile agar medium (*Oxoid*) and the plates were incubated at 30°C for 18hr. Potato dextrose agar medium was used for the enumeration of yeasts and moulds and incubated at 25°C for 5 days. Total bacteria count (TBC) was determined using plate count agar medium. Plates were incubated at 32°C for 48hr. The colony counted from each plate was calculated using the following formula.

$$CFU/g = \left(\frac{\text{average number of colonies from duplicate plates}}{\text{dilution factor x volume plated}}\right)$$

Plating and Isolation of Lactic Acid Bacteria

Plating has been done within 3-6 h of arrival of the samples at the laboratory. According to the method described by Richardson (1985), Enumeration of lactic acid bacteria was done after plating 0.1 ml of 10^{-1} to 10^{-5} dilution of the samples onto de Mann Rogosa Sharp agar (MRS) (HiMedia). The agar plates were incubated in an anaerobic jar (BBL, GasPak Plus) at 35^oC for

48 h with replications. Ten colonies randomly selected from each clear and countable MRS agar plate and purified by successive streaking onto MRS agar, before being subjected to characterization as described by Esayas *et al.* (2008).

Characterization of Lactic Acid Bacteria

According to the method described by Esayas *et al.* (2008), from each plate, five pure and clear colonies were selected based on their colony morphology. The colonies then were inoculated into MRS broth, incubated at 35^{o}C for 12 h, and then maintained in the refrigerator at 4^{o}C for further characterization. The isolates were identified as lactic acid bacteria using gram staining, colony and cell morphology, and catalase reaction test. Additionally, the isolates were characterized based on their physiological tests like growth on 2%, 3%, 4%, 6.5%, and 10% NaCl concentrations, and growth at 10^{0}c, 37^{0}c and 45^{o}C temperature. Further, the isolates were identified at species level using biochemical test such as gas production from glucose fermentation, and sugar fermentation profiles as described by Bergey (2009). Isolates that were characterized as lactic acid bacteria were kept in glycerol stock cultures in the refrigerator at -20^{o}C for further analysis (Table 2).

Table 2: Types of test to characterize isolated of lactic acid bacteria.

No	Types of test	characteristics
1	Morphological test	Cell morphology Cell arrangement
2	Biochemical test	Gram stain Catalase test Gas production, Sugar fermentation
3	Physiological test	Growth in NaCl conc. Growth at different temperature

(Eyassu Seifu *et al*, 2012)

Antimicrobial Activity Test

Clinical isolates of *S. aureus* (ATCC-25923), *E. coli* O157:H7 (ATCC-25922), *Listeria monocytogen* and *Salmonella typhi* were taken from the Ethiopian Public Health Institute, Addis Ababa, and were used as test microorganisms. The antimicrobial activities of the isolates were determined by using the disc diffusion procedure as described by Savadogo *et al*. (2004) and Girum *et al*. (2005). A well-isolated colony was selected from MRS agar plate. Based on the European Committee for Antimicrobial Susceptibility Testing protocol (EUCAST, 2000), the microbial suspension containing 10^7 CFU/ml was prepared by taking active LAB colonies with a sterile loop and dipped into sterile distilled water until it becomes similar in color with 0.5% McFarland solution. The suspension was centrifuged (10, 000 rpm for 20 min at 4^oC) to obtain the cell free culture supernatant as described by Esayas *et al*. (2008). The pH of the culture supernatant was adjusted to pH 7 with 1 M NaOH to exclude the antimicrobial effects of organic acids (Savadogo *et al*., 2004). Two controls were prepared using un-inoculated MRS broth for negative control and Nisin (100 mg/ml) for positive control.

A sterile cotton swab was dipped into a culture of the test (indicator) microorganism, which had been grown in Tryptone Soya Broth (Oxoid) for 24 h at 35^oC. The swab rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculums. The dried surface of Mueller-Hinton agar (HiMedia) inoculated by streaking the swab over the entire agar surface. This procedure was repeated by streaking two more times, rotating the plate each time to ensure an even distribution of inoculums. Sterile filter discs of 6 mm in diameter were prepared from Whatman filter paper no. 1. Using micropipette, 5 µl of the culture supernatant was added to each disc (Lalitha, 2005). Four discs (with culture supernatant)

were placed on a 100-mm plate. Each plate was incubated at 35^{o}C for 24h and examined by measuring the diameter of the inhibition zones digital calipers. Then, the numbers was recorded to the nearest whole number.

3.3. Data analysis

Each physiochemical and microbiological count raw data was recorded on a data sheet table and then it was analyzed using one way ANOVA. Statistical Analysis System (SAS) version 9.0 for windows (SAS 2008) was used for data analysis. All laboratory experiments were repeated two times. The physicho-chemical and microbiological data were compared by performing a one-way analysis of variance on the replicates at 95% level of significance, Significant results refer to $P<0.05$.

3.4. Statistical model:

The statistical model will be:-

$Yij = \mu + ai + \varepsilon ij$

Where:

Yij= individual observation (TBC, TCC, yeast and mould count, LAB and composition of *Shimpia*)

M= overall mean

Ai= agro ecology i=3, (highland, midland, lowland).

Eij= error term

4. RESULT AND DISCUSSION

4.1. Socio-economic Characteristics of the Study Area

House hold characteristics

Majority of the respondents (67.28%) under the current study were female (Table 3).The highest proportions 98.77% of them were married. This result is higher than the report made by Haile *et al.* (2012) who indicated that 47.7% of the participants were females in Hawassa city. The average household family size was 8.1 ± 0.3. The overall average age of respondents was 43.4 ± 1.3 with majority (86.2%) of them were aged between18 and 55 years. .This indicated that the existence of high potential of productive labor force which can be used as production input to produce more *Shimpia* and other dairy products in the study area. Age and family size results in the current study are different from results reported by Tenagnework (2016) who indicated that family size was 6.4 ± 0.3 with high proportions (96.7%) of age groups between18 and 55 years in *Guji* zone, *Oromia* region.

The educational status of respondents demonstrated that, 49.38% of them were unable to read and write, 17.28% of the them were able to read and write, nearly 26% of them had attended primary school education and the rest were high school and above.

Demographic characteristics are nearly similar through agro ecologies except that there is difference with educational level of households among agro ecologies. Thus the highest percentage (31.7%) of the household's respondents who attended at elementary level was in midland (*Bossakecha kebele*). This may be due to the location of the area (*Bossakecha kebele*) which is the nearest to *Wollayta Sodo* town which provides households with better chance to educational access.

About half of the respondents were non-educated which may attribute poor milk handling practice as described by Dehinenet *et al., (2013),* who stated that educational status of milk producers is major determinant factor for milk safety.

Table 3: Demographic characteristics of respondents across agro ecologies in *Sodo Zuria* district

Variables	**Respondents**			
	Highland (n= 79)	Midland (n=45)	Lowland (n= 38)	Over all (n= 162)
Gender (%)				
Male	32.9	33.3	31.6	32.72
Female	67.1	66.7	68.4	67.28
Age (M±SE)	45.1± 0.6	43.2 ± 0.2	41.9 ± 0.9	43.4 ±1.3
Family size (M±SE)	7.8 ± 0.3	8.3 ± 0.5	8.2 ± 0.1	8.1 ± 0.3
Marital status (%)				
Married	98.7	97.8	100	98.77
Single	1.3	2.2	0	1.23
Educational level (%)				
Can't read and write	49.4	43.9	52.4	49.4
Read and write	17.7	17.8	18.4	17.3
Elementary	25.3	31.7	21.3	25.9
High school	5.1	4.4	5.3	4.9
>High school	2.5	2.2	2.6	2.5

*Highland = *Kokatemarachere*, midland= *Bosakecha,* lowland= *Gulgula kebeles: HHs*

4.2. *Shimpia* Production

Respondents stated that they used whole cow milk sourced from their own cow to produce *Shimpia*. The majority (57.9%) of the respondents had local breed of cows. Whereas, about, 28.2% of respondents had crossbreed cows (Local x Holstein Frisian) at varying exotic blood level while the nearly (14%) of the respondents had both local and cross breed cows (Table 4).

Among the respondents, the highest proportion (37.5%) of them who used milk sourced from crossbred cows (Local x Holstein Frisian) was found in highland area. This may be due to the favorable agro ecology of highland for adaptation of crossbreed cows.

The source of water used for cleaning of milk and milk product handling equipment including *Shimpia*, was tap water as indicated by the majority (64.8%) of the respondents followed by ground water (17.9%) from their own hand-dug well and the rest of them (17.3%) used spring water. The highest percentage of HH's (73.2%) that used tap water were in the midland area and this may be due to the proximity of the area to *Wolayta Sodo* town which has better access to tap water.

All of the households clean their equipment used for *Shimpia* processing before and after processing by using different cleaning detergents and plants. About 52.5% of them used local herbs only for cleaning. The most commonly used herbs according to the respondents are *Kosoret (Lippia adoensis)* and *Bahirzaf (Eucalyptus globules Labill)*. About 40.1% of respondents used commercially available cleaning detergents (Ajax and Omo). Approximately 7.4% of respondents used both local herbs and commercial cleaning agents simultaneously (Table 4). The cleaning habit and cleaning agents is almost similar across the three agro ecologies (highland, midland and lowland). In line with this findings, in Ethiopia, different locally available herbs used by traditional milk and milk product producers for cleaning their milk handling equipment and are considered important to keep their products longer and provide good odor and flavor (Tesfemariam *et al*, 2017).

The use of local herbs as cleaning agents of milk handling equipment by most of the households (HH's) can have its own effect on the sensory (flavor) and microbiological qualities of *Shimpia* and other milk products. Thus, some herbs like *Kosoret (Lippia adoensis)* can inhibit growth of

pathogenic undesirable microorganisms in milk and milk products (Tewodros and Muluken, 2018)

Breed difference of cows from which milk is collected for *Shimpia* production can affect the composition of *Shimpia*. This is because breed is the determinant factor for the composition of milk (Dehinenet *et al., 2013*). The use of spring and ground (hand dug well) water for milk handling equipment cleaning purpose by some of the households (HH's) can affect the microbiological quality of *Shimpia* and other milk products. Spoilage and pathogenic microorganisms in milk and milk products can be sourced from cleaning water obtained from un hygienic source (Haile *et al.*, 2012).

Table4: Milk source, cleaning practices of milk and *Shimpia* handling equipment in the study area

Production factors	**Agro-ecologies**			
	Highland(n=79)	Midland(n= 45)	Lowland(n=38)	Over all(n=162)
Milk source (%)				
local breed cows	49.5	60.0	60.5	57.9
Cross breed cows	37.5	26.7	25.3	28.2
Both local and cross breed cows	12.7	13.3	14.2	13.9
Cleaning Water source (%)				
Groundwater (hand-dug well)	10.1	9.0	31.5	17.9
Tap water	62.2	73.2	60.1	64.8
Spring water	27.7	17.8	8.4	17.3
Cleaning agents (%)				
commercial detergents only	40.5	40.0	39.5	40.1
Local herbs only	51.9	53.3	52.6	52.5
Both commercial detergents and herbs	7.6	6.7	7.9	7.4

*Highland = *Kokatemarachere*, midland= *Bosakecha*, lowland= *Gulgula kebeles*

Shimpia processing technique

According to the current study, households can make *Shimpia* is up to three times per week or as they need. During *Shimpia* preparation, first they pour milk into clay pot which is previously cleaned, dried and fumigated with *Woyra (Olea africana)* or *Tid* (*Juniperus procera)* wood. The clay pot holding the milk was covered with *coba* (*Musa sapientum)* leaf and left for natural fermentation for about 24-26 hours depending on the weather until it forms curdle. Once the milk curdles, they tightly cover the fermentation pot with *coba* (*Musa sapientum)* leaf and tie with fiber rope to avoid outflow of milk during churning. The content is churned for about 15-20 minutes until the mass foams and they observe very fine butter grains through sight hole pored at the neck of the pot for check up. The butter grains are very small and cannot be harvested as butter except that they are visible with naked eyes. Besides their observation *Shimpia* producers sometimes use the texture of milk shake as reference to call it *Shimpia.* They take sample through site hole and check the texture with their fingers. Smooth texture and foamy appearance is desirable to harvest it as *Shimpia*. Finally they pour out certain amount of foamy fermented milkshake immediately after they obtained fine (small size) and evenly distributed butter grains which is called *Shimpia.* Churning processes continue until the remaining butter milk (i.e., the amount left from *Shimpia*) recovered to traditional butter and local *ayib* (Figure 1). *Shimpia* can be served immediately after preparation or otherwise can be kept for about 30 minutes at room temperature. As it stays longer it develops sour taste which is not desirable by consumers.

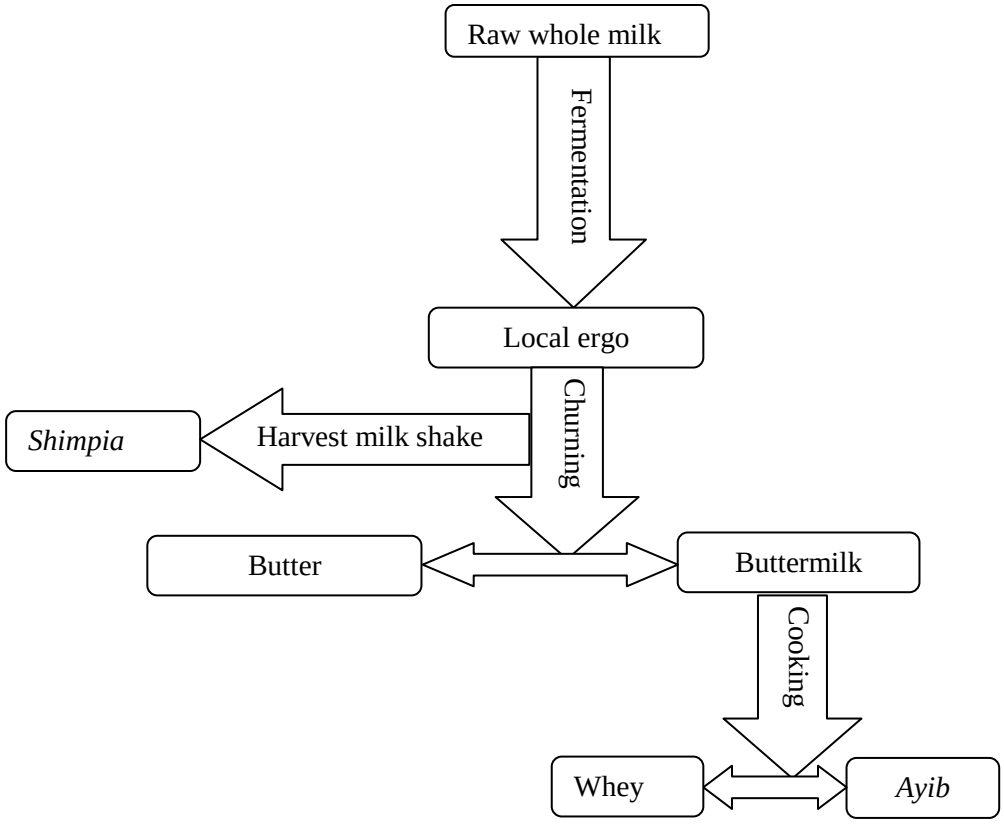

Figure 1.*Shimpia* processing flow chart

4.3. Physicochemical analysis of *Shimpia*

The pH of *Shimpia* ranged from 4.5 to 4.7 (Table 5) and this result was slightly higher than the pH values obtained for *ergo* (traditional fermented sour milk) samples, 4.3 to 4.5 reported by Zelalem (2012). The difference might be due to the agro- ecological difference of the study area and fermentation period (Almaz, 2017; and Assefa *et al.*, 2008).The low pH level of *Shimpia* might be due to the production of lactic acid by fermentative microbes during spontaneous fermentation of milk for *Shimpia*. There was no difference ($P>0.05$) in pH of *Shimpia* between the three agro- ecologies.

The similarity in pH found across the selected agro-ecology could be due to the practice of back-sloping technique in the highland area (kokate-Marachere kebelle). Thus ambient temperature in the highland is relatively low and the growth of LAB is also relatively slow. However, when using a portion of *ergo* from a previous batch as a starter is a common practice (back-slopping) can fasten the processes of fermentation. On the other hand, this technique is not commonly practiced in the low land are due to sufficient amounts of LAB that can proliferate on the inner walls of the container and can grow better by the help of warmer ambient temperature. Back-slopping is also common in midland areas during cold season. This is in line with the report of Assefa (2008) that indicates the environmental condition could affect the fermentation process of *Ergo.*

The average percentage constituents of *Shimpia* are total solid 11.7%, fat 4.5%, protein 3.1%, lactose 3.7 and ash 0.7%. All results where different from the composition of *ergo* reported by Muluken *et al*.(2016) who reported total solid 33.38%, fat 6.2, protein 7.0 and ash 1.41.There was a significant difference($p<0.05$) between the fat and total solid contents of *Shimpia* from different *agro ecologoes*. The difference with total solid of *Shimpia* could be due to difference with its fat content. The highest fat content (4.95%) was obtained from highland (*Kokate-marachere)* while the lowest (4.10%) was from lowland (*Gulgula).* The difference might be due to the agro-ecological difference which can affect the feed type and availability and the intake which also can affect milk fat content (Ferlay *et al*., 2006).There was no difference (*P>0.05)* between the protein, lactose and ash contents of *Shimpia* among different agro ecologies in the study area.

Table 5: Compositional quality and pH of *Shimpia* samples collected from the study area

Agro– ecology	Composition and pH					
	Fat	**Protein**	**Lactose**	**Total solid**	**Ash**	**pH**
Highland	4.95±0.07 [a]	3.10±0.18	3.71±0.20	12.20±1.2[a]	0.70±0.08	4.51±0.03
Midland	4.45±0.13[ba]	3.20±0.09	3.70±0.32	11.70±1.5[ba]	0.75±0.09	4.50±0.01
Lowland	4.10±0.12 [b]	3.10±0.01	3.71±0.20	11.15±1.1[b]	0.70±0.06	4.53±0.06
Average	4.5±0.08	3.1±0.16	3.7±0.27	11.7±1.1	0.7±0.07	4.5±0.02

*Mean value ± SE of 3 agro ecologies, with different superscripts within the same column are significantly different ($p<0.05$).
*Highland = *Kokatemarachere*, midland= *Bosakecha*, lowland= *Gulgula kebeles*

4.4. Microbial Analysis of *Shimpia*

The average microbial load of *Shimpia* prepared at farmers' house is 8.79±0.04 for total aerobic bacteria count, 6.49±0.05 for yeast and mould count, 4.77±0.01 for total lactic acid bacteria, and 4.22±0.03 for total *coliform* count $\log_{10}$ cfu/g (Table 6). Total bacteria count and yeast and mould count in this study are different from the result reported by Zelalem (2012) for microbial analysis of *Ergo* who reported 9.49 for total bacteria count, 4.51 for total *coliform* count, and 8.38 for yeast and mould count $\log_{10}$ cfu/g. However, *coliform* count was almost similar in the two studies. The difference with total bacteria count and yeast and mould might be due to the agro-ecology difference of areas where the samples were collected. This is because ambient temperature can affect the growth and type of microorganisms (Assefa *et al*., 2008).

In this study there was no significant difference ($p>0.05$) among *agro ecologies* with results of yeast and mould counts. The similar prevalence of yeast and mould in different agro-ecologies might be due to the practice of back-sloping in highland areas and sometimes in mid-land agro-

ecologies during cold season. The addition of sour *ergo* from previous batch as a starter causes inoculates high load of yeast and mould which are resistant to low pH, where as other deteriorative and pathogenic bacteria are less resistant and less prevalent in sour milk (Almaz *et al*., 2001; and Savadogo *et al.*,2004). On the other hand there is significant difference ($p<0.05$) among *agro ecologies* with results of total aerobic bacteria, lactic acid bacteria and *coliform* counts. This difference might be due to the differences observed in the educational status, milk handling practices, type of water used for milk utensils cleaning, cleaning agents used and fumigation practices of farmers. All these conditions can affect the types and load of microorganisms in raw milk and hence reasons for variation of total aerobic bacteria and *coliform* counts (Abdulkadir *et al*., 2011; Ashenafi and Fikadu, 1994 and Tesfemariam *et al*., 2017).

Table 6: Microbial analysis of *Shimpia* from different agro ecologies in *sodozuria* District

Agro ecology	**Microbiological parameters**			
	TBC	**Y&M**	**TLAB**	**Coli form**
Highland	8.06 ± 0.06^{c}	6.40 ± 0.06	4.61 ± 0.05^{b}	3.75 ± 0.08^{b}
Midland	8.63 ± 0.01^{b}	6.48 ± 0.04	4.76 ± 0.02^{a}	3.95 ± 0.02^{b}
Low land	9.69 ± 0.04^{a}	6.59 ± 0.04	4.74 ± 0.04^{a}	4.97 ± 0.02^{a}
Average	8.79 ± 0.04	6.49 ± 0.05	4.77 ± 0.01	4.22 ± 0.03

*Mean value ± SE of 3 agro ecologies, with different superscripts within the same column are significantly different ($p<0.05$)

*TBC= total aerobic bacteria count, Y&M=yeast and mould, TLAB= total lactic acid bacteria.

*Highland = *Kokatemarachere*, midland= *Bosakecha*, lowland= *Gulgula kebeles*

4.5. Isolation of lactic acid bacteria

Lactic acid bacteria grown on MRS agar media were identified from *Shimpia* samples by using biochemical tests like gram staining, catalase test, cell shape, cell arrangement. Based on these, bacteria that are Gram-positive, catalase-negative, cocci, cocco-bacilli or rod-shaped isolates with characteristic cell arrangements when grown on MRS agar medium were considered as lactic acid bacteria (Esayas *et al*., 2008 and Savadogo *et al*., 2004). From the total of 150 bacteria isolated from 30 *Shimpia* samples, 72 were considered as lactic acid bacteria while the remaining 78 were non lactic acid bacteria (Table 7). From the 72 lactic acid bacteria types, 23 were from midland (Bossakecha), 18 from highland (Kokatemarachere) and 31 from lowland (Gulgula) kebeles. The lactic acid bacteria isolated from *Shimpia* comprised of six genera. These are *lactobacillus, streptococcus, pediococcus, leuconostoc, lactococus* and *Entrococus*. The result is slightly different from the report by Assefa *et al.*(2008) reported as five LAB genera were identified from *Ergo* that included *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Entrococcus* and *Streptococcus*. The difference is *pediococus* did not exist in *ergo* while it existed in *Shimpia* and it might have gotten in to the milk form *coba* or *inset* (*Musa sapientum)* leaf used for covering of churning pot during *Shimpia* processing. *Coba* leaf (*Musa sapientum)* is the common habitat for *pediococus* (Michael, 2008).

There was difference among *Shimpia* samples from different *agro ecologies* with type and load of lactic acid bacteria. This shows the variation of *Shimpia* at different locations or agro-ecology with its microbiological profile. This is in agreement with the reports by Savadogo *et al.*, (2004), Ayad *et al*., (2004) and Esayas *et al*., (2008).

Table 7: Lactic acid bacteria (LAB) isolated from *Shimpia* collected from *Sodozuria District*

agro	**Genera of Lactic Acid Bacteria**						
ecology	***lactobacillus***	***streptococcus***	***pediococcus***	***leuconostoc***	***lactococus***	***Entrococus***	Non-LAB
Highland	7	2	4	0	2	3	29
Midland	9	1	4	2	2	5	25
Lowland	11	5	2	3	3	7	24
Total	27	8	10	5	7	15	78

*Number of genera of LAB from different *s* with different agro-ecology,

*Highland = *Kokatemarachere*, midland= *Bosakecha*, lowland= *Gulgula kebeles*

Among the isolates of LAB that can potentially inhibit pathogenic bacteria, the genera *lactobacillus* is the major one with characteristics rod medium long cell shape, chain and pair cell arrangement, unable to produce CO_2 from glucose, unable to grow at temperature of 10 ^{0}C while they grow at 37^{0}c and 45^{0}c and no growth in NaCl concentrations 6.5% and 10% while they grow at 2%, 3%and 4% concentrations (Table 8). LAB with characteristics cocci cell shape, chain cell arrangement, unable to produce CO_2 from glucose, unable to grow at temperature 10^{0}c while they grow at 37^{0}c and 45^{0}c, grow in NaCl concentrations 2% and 3% while unable to grow in 4%, 6.5% and 10% concentrations is categorized as genera *streptococcus.* The other genera is *pediococcus* with characteristics *cocci* cell shape, chain cell arrangement, unable to produce CO_2 from glucose, unable to grow at temperature 45 ^{0}c while they grow at 37^{0}c and 10^{0}c, unable to grow in NaCl concentrations 2%, 3%, 4%,6.5% and 10%. The genera *leuconostoc* is characterized by *cocci* cell shape, chain cell arrangement, produce CO_2 from glucose; grow in 2% and 3% while unable to grow in 4%, 6.5% and 10% NaCl concentrations.

Table 8: Morphological, biochemical and physiological characteristics of LAB isolated from *Shimpia* and are able to inhibit pathogenic bacteria.

LAB Culture characteristics	**LAB isolates**							
	BK-11	BK-12	BK-13	BK-14	KM-21	KM-22	GG-24	GG-42
Morphological tests								
Cell shape	R & ML	R &ML	R &ML	R & ML	**C**	**C**	**C**	**C**
Cell arrangement	Ch&p	ch	ch	Ch	ch	ch	ch	ch
Biochemical tests								
Gram stain	+	+	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-	-	-
Physiological tests								
CO_2 from glucose	-	-	+	-	-	-	+	+
Growth at								
10^0c	-	-	+	+	-	+	+	-
37^0c	+	+	+	+	+	+	+	+
45^0c	+	+	+	+	+	-	-	-
Growth in NaCl								
2%	+	+	+	+	+	-	+	+
3%	+	+	+	+	+	-	+	+
4%	+	-	+	+	-	-	-	+
6.5%	-	-	+	-	-	-	-	+
10%	-	-	-	-	-	-	-	-
Identified genera	Lb	Lb	Lb	Lb	**Sc**	Pc	Leuc.	Leuc.

*+= growth detected; - =no growth; R & ML = rod and medium long; C = cocci; Ch &P = chains and pairs;ch= chain; Lb= *lactobacillus*; Sc= *streptococcus*; Pc= *pediococus* and Leuc.= *leuconostoc.*

LABs that are able to inhibit pathogenic bacteria are identified at species level by using biochemical and carbohydrate fermentation profile (Table 8 and 9). LAB with characteristics rod medium long cell shape, chain and pair cell arrangement, unable to produce CO_2 from glucose, unable to grow at temperature 10 ^{0}c while they grow at 37^0c and 45^0c and no growth in NaCl concentrations 6.5% and 10% while they grow at 2%, 3%and 4% concentrations, able to ferment lactose, maltose, sucrose and xylose while unable to ferment manitole characterized as *L.delberki ssp.lactice.* LAB with characteristics rod medium long cell shape, chain and pair cell arrangement, unable to produce CO_2 from glucose, unable to grow at temperature 10 ^{0}c while they grow at 37^0c and 45^0c and no growth in NaCl concentrations 6.5% and 10% while they

grow at 2%, 3%and 4% concentrations, able to ferment lactose, sucrose, and xylose while unable to ferment maltose and manitol are characterized as *L.dulburikissp.indicus.* under the genera of lactobacillus species with characteristics rod medium long cell shape, chain and pair cell arrangement, unable to produce CO_2 from glucose, unable to grow at temperature 10 ^{0}c while they grow at 37^{0}c and 45^{0}c and no growth in NaCl concentrations 6.5% and 10% while they grow at 2%, 3%and 4% concentrations, able to ferment lactose, sucrose, maltose xylose while unable to ferment manitol, is *L.panis.* LAB species that are rod medium long cell shape, chain and pair cell arrangement, produce CO_2 from glucose, unable to grow at temperature 10 ^{0}c while they grow at 37^{0}c and 45^{0}c and no growth in NaCl concentrations 6.5% and 10% while they grow at 2%, 3%and 4% concentrations, able to ferment lactose, sucrose, maltose xylose while unable to ferment manitol, is *L.gasseri.* LAB with characteristics cocci cell shape, chain cell arrangement, unable to produce CO_2 from glucose, unable to grow at temperature 10 ^{0}c while they grow at 37^{0}c and 45^{0}c, grow in NaCl concentrations 2% and 3% while unable to grow in 4%,6.5% and 10% concentrations, able to ferment lactose, sucrose, maltose xylose while unable to ferment manitol, is *S.agalactiae.* LAB species with characteristics *cocci* cell shape, chain cell arrangement, unable to produce CO_2 from glucose, unable to grow at temperature 45 ^{0}c while they grow at 37^{0}c and 10^{0}c, unable to grow in NaCl concentrations 2%, 3%, 4%, 6.5% and 10%, unable to ferment sucrose and ferment lactose, maltose, xylose and manitol is *P.cellicolla.* LAB under the genera *Leuconostoc* characterized by *cocci* cell shape, chain cell arrangement, produce CO_2 from glucose, grow in 2% and 3% while unable to grow in 4%,6.5% and 10% NaCl concentrations however, is able to ferment lactose, sucrose, maltose and xylose while unable to ferment manitol is *Leuc.Mesenteroide ssp. dextranicum.*LAB under the genera *Leuconostoc* characterized by *cocci* cell shape, chain cell arrangement, produce CO_2 from glucose, grow in

2% and 3% while unable to grow in 4%,6.5% and 10% NaCl concentrations able to ferment lactose, sucrose, maltose and xylose and manitol is *Leuc.mesenteroides ssp. Mesenteroides*

Table 9: Sugar fermentation profiles of LAB isolated from *Shimpia* and are able to inhibit pathogenic bacteria

sugars	**LAB isolates**							
	BK-11	BK-12	BK-13	BK-14	KM-21	KM-22	GU-24	GU-42
Lactose	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	-	+	+
Maltose	+	-	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+
Manitol	-	-	-	-	-	+	-	+

BK-11 = *Lb.delberkissp.lactice;BK- 12= Lb.dulburikissp.indicus; BK-13=Lb.panis; BK -14=Lb.gasseri; KM-21= S.agalactiae; KM-22=P.cellicolla;GG-24=Lc.mesenteroidesssp.dextranicum; GG-42=Lc.mesenteroidesssp.mesenteroides ;*

+ Able to ferment;

− Unable to ferment.

4.6. Antimicrobial activity of the isolates

The disc diffusion test for the determination of the antimicrobial activity of LAB isolate is expressed as potentially antagonist with inhibition zone diameters mm against these indicator microorganisms, (Table 10). From a total of 72 lactic acid bacteria isolate 8 strains showed effective antibacterial effect when tested against different pathogenic test strains. The diameters of inhibition zone ranged from 9mm to 11.8 mm. The biggest inhibition zone (11.8mm) was obtained from the culture supernatant of lab strain KM-22 (*pediococcus cellicolla)* on the test strain *Salmonella typhi.* This LAB strain is also able to inhibit *Listeria monocytogenes and E. coli* with inhibition zone diameter of 9.5 and 9.0 mm respectively**.** GG-42 (*Lactococcus mesenteroides ssp. Mesenteroides)* could inhibit *Salmonella typhi, S.aurious* (ATCC-25923) *and Listeria monocytogenus* with inhibition zone diameter of 9.5mm, 11mm, and 9mm respectively. On the other hand *Listeria monocytogenus* is the most sensitive pathogen that was inhibited by

eight LAB strains. In contrast *E. coli* O157:H7 (ATCC-25922) was the most resistant strain that could be inhibited by only one LAB strain (*Pediococcus cellicolla).* Similarly Girum *et al*. (2005) and Esayas *et al*. (2008) reported different inhibition zone diameter by testing different LAB strains against different test pathogenic bacteria. This is because different LAB strains produce different type and amount of antimicrobial substance that can act differently on pathogenic bacteria strains (Savadog *et al*., 2004).

Table 10: Average diameter of inhibition zones and antimicrobial activity of the inhibitory culture supernatant, on some indicator strains, using the disc diffusion assay (Mean ± SD)

No	LAB strain	Test Bacteria	Inhibition zone Diameter mm
1	BK-11	*Salmonella typhi*	9±0.00
		Listeria monocytogenes	10±0.01
2	BK-12	Listeria monocytogenes	11±0.10
3	BK-13	Listeria monocytogenes	9.5±0.00
4	BK-14	*Listeria monocytogenes*	11±0.21
5	KM-21	*Listeria monocytogenes*	9.5±0.30
6	KM-22	E. coli O157:H7	9±0.00
		Salmonella typhi	11.8±0.03
		Listeria monocytogenes	9.5±0.07
7	GG-24	*S,aurious* (ATCC-25923)	9±0.12
		Listeria monocytogenes	10.5±0.10
8	GG-42	*Salmonella typhi*	9.5±0.00
		S,aurious (ATCC-25923)	11±0.24
		Listeria monocytogenes	9±0.61

In this study gram positive bacteria were more sensitive to antimicrobial effect of many LAB strains than gram negative strains. The sensitivity of gram positive bacteria is due to the nature of their cell wall. Thus pediocin (bacteriocin produced by *Pediococcus acidilactic*) interacts with

lipoteichoic acids that exist in gram-positive bacteria, where as it is absent in gram negative bacteria (Esayas *et al*. 2008). In addition most nisin producer LAB strains are effective to inhibit gram-positive pathogenic bacteria while they show less effect on gram negative pathogenic bacteria. This is because of the antimicrobial action of nisin is on the cell wall of bacteria and this makes it effective on gram-positive pathogenic bacteria (Soormro *et al.*, 2002, and Osama. *et al*. 2019).

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusions

Shimpia is traditional fermented milk shake produced from naturally fermented whole cow milk among Wolayta community. It is produced for home consumption only to be provided for respected and lovely family members and special gusts to show them respect and love. Even though the traditional *Shimpia* processing techniques are similar by all producers, there are differences with cleaning and fumigation practices among different communities.

Like most of other traditional dairy products in Ethiopia, *Shimpia* contains variety of microorganisms. Besides the existence of microorganisms that are indicator for contamination. There are important lactic acid bacteria in *Shimpia* that are identified under the current study. Six genera of lactic acid bacteria exist in *Shimpia* with varying load and type in different agro-ecologies. The dominant genus of lactic acid bacteria in *shimia* is lactobacillus. Eight lactic acid bacteria strains that belong to four genera were isolated from *Shimpia* with proven potential microbes to inhibit pathogenic bacteria. The LAB isolates showed more inhibition effect against gram-positive pathogenic bacteria than gram-negatives. Thus *listeria monocytogenes* was the most susceptible while *E. coli* O157:H7 was the most resistant.

5.2. Recommendations

Based on the above conclusion the following points are recommended.

- Since *Shimpia* is highly acceptable traditional dairy product among *Wolayta* community, studies need to be conducted to assess the market potential to involve *Shimpia* in traditional dairy products and to adopt it by other communities.
- Studies should be conducted to produce *Shimpia* in hygienic and controlled fermentation for commercial purpose by keeping its original sensory and nutritional identities.
- Use of cooling facilities should be used to extend the shelf life of *shimpia*.
- Valuable microorganisms found in *Shimpia,* other than lactic acid bacteria should be studied in detail in line with their contribution for sensory and nutritional values.
- Lactic acid bacteria in *Shimpia* should be further studied at molecular level for high certainty of identification.
- The potential of Lactic acid bacteria in *Shimpia* as starter culture and probiotics need to be studied further.
- Antibacterial components produced by lactic acid bacteria should be characterized and their potential effect in different pH and temperature ranges to be used in food processing line in place of artificial preservatives should be looked at.

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Annex I: Questionnaires

Questioners for the survey components of MSC thesis study on

CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF LACTIC ACID BACTERIA ISOLATED FROM *SHIMPIA* (AN ETHIOPIAN TRADITIONAL FERMENTED MILK PRODUCT), AGAINST SELECTED PATHOGENIC MICROORGANISMS

Note for Enumerator:

Introduce yourself and start by recognizing their willingness and time to respond for the interview. Explain objective of the study, how they are selected and use of data:

- Aim of conducting the study is to know physical, chemical and microbiological quality of *Shimpia* and required interventions to engage the product in commercial level in other community.
- You are selected randomly from small scale milk house operators.
- The information collected from you will be used as a summary to inform the study
- You are free to ask clarification questions or not to respond questions which you are not comfortable.

Interviewer Reference

- Assistant researcher name: ----------
- Lead Consultant ----------
- Date: ---------- Time ----------
- Questionnaire no/Code ----------

Respondent General Information

- Name of Respondent:_____
- Sex of the respondent:
  - Male
  - Female
- Age of the respondent: _____ years
- Name of region _____ Zone _____ woreda _____ kebele_____

- Current occupation of the respondent _____
- Telephone ----------

Dairy products production at home

1. What type of products do you produce at home?
    - Local *ergo*
    - Liquid milk
    - Local *ayib*
    - *Shimpia*
    - All
    - Others
        - ----------
        - ----------
        - ----------
2. For what purposes you produce dairy products at home?
    - For home consumption only
    - For market only
    - For both
3. Do you produce Shimpia for market?
    - Yes
    - no

3.1 If yes who are your customers?

- Individual consumers
- Hotels/ cafes
- All
- Others

3.2 How much is the cost of *Shimpia* per liter? ----------

3.3 If no why? ----------

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4. When do you produce *Shimpia*? ----------

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5. For whom do you prepare Shimpia at home?

- For all family members
- For respected gust
- For lovely child
- For parents
- others

***Shimpia* processing technology**

1. What type of milk do you use to make *Shimpia*?
   - Whole milk
   - Skimmed milk
2. What types of equipments do you use and their use?
   - ----------to----------
   - ----------to----------
   - ----------to----------
   - ----------to----------
   - ----------to----------
3. When do you clean equipments for Shimpia processing?

- Before processing
- After processing

4. how do you clean them?
   - By using cold water
   - By using hot water
   - By using detergents
5. What types of cleaning agents (detergents) do you use?
   - Powder soap (Omo)
   - Bar soap (ajaks)

- Berekina
- Any other

6. What is your source of water for cleaning and other use?
    - Ground water
    - Common potable water
    - River or spring water
7. Do you use any additives during *Shimpia* processing?

    7.1 If yes what types of additives?

    - ----------
    - ----------
    - ----------
    - ----------

8. Do you apply fumigation of equipments to be used for *Shimpia* processing?

- Yes
- no

    8.1 If yes what types of wood do you use for smoking?

    - ----------
    - ----------
    - ----------

9. For how long do you ferment the milk for Shimpia processing?

    9.1 During hot season ----------

    9.2 During cold season ----------

10. For how long do you store *Shimpia after production*?

    10.1 In fridge ----------

    10.2 In room temperature ----------

11. How much volume of Shimpia do you get from one liter of milk?
    - ----------
12. What is your raw milk source for Shimpia production?
    - Your own farm milk
    - other farmers

- Middle men
- Others

13. What is the breed of the cows sourced milk for Shimpia?
- Cross breed
- Local breed
- Both
- unknown

14. Do you face any quality defects on Shimpia?
    - Yes
    - No

15. If yes what types of defects you faced?
- ----------
- ----------
- ----------
- ----------

General recommendation

1. What do you recommend to produce quality Shimpia and engage it in advanced market and make it popular in other areas of Ethiopia?

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2. Any other comment ----------

Thank you for your time and very valuable input!

Annex II: biochemical and physiological test result table

LAB isolates		Type of Tests		
	Cell shape	**Cell arrangement**	**Gram stain**	**Catalase Test**
BK-11	R & ml	Ch & P	+	-
BK-12	R & ml	Ch	+	-
BK-13	R & ml	Ch	+	-
BK-14	R & ml	Ch	+	-
BK-15	R & ml	Ch	+	-
BK-21	C	Ch	+	-
BK-22	C	Ch	+	-
BK-23	C	Ch & P	+	-
BK-24	R & ml	Ch	+	-
BK-25	C	Ch	+	-
BK-41	c	Ch	+	-
BK-42	R & ml	Ch	+	-
BK-45	R & ml	Ch	+	-
BK-54	R & ml	Ch & P	+	-
BK-61	C	Ch & P	+	-
BK-63	C	Ch & P	+	-
BK-65	C	Ch	+	-
BK-72	R & ml	Ch	+	-
BK-75	C	ch	+	-
BK-93	C	Ch & P	+	-
BK-101	C	Ch & P	+	-
BK-103	R & ml	Ch & P	+	-
BK-104	C	Ch & P	+	-
KM-14	C	Ch & P	+	-
KM-15	C	Ch & P	+	-
KM-21	C	Ch	+	-
KM-22	C	Ch	+	-
KM-44	R & ml	Ch & P	+	-
KM-45	R & ml	Ch & P	+	-
KM-51	C	Ch	+	-
KM-54	C	Ch	+	-
KM-61	C	Ch	+	-
KM-62	C	Ch	+	-
KM-63	C	Ch	+	-
KM-72	C	Ch	+	-
KM-74	C	Ch	+	-
KM-81	C	Ch & P	+	-
KM-82	C	Ch & P	+	-
KM-85	C	ch	+	-

KM-102	C	Ch & P	+	-
KM-104	R & ml	Ch & P	+	-
GU-13	C	Ch	+	-
GU-14	R & ml	Ch & P	+	-
GU-15	R & ml	ch	+	-
GU-21	C	Ch & P	+	-
GU-22	R & ml	Ch & P	+	-
GU-23	R & ml	Ch & P	+	-
GU-24	C	Ch	+	-
GU-32	R & ml	Ch & P	+	-
GU-33	R & ml	Ch & P	+	-
GU-35	C	Ch & P	+	-
GU-41	C	Ch & P	+	-
GU-42	C	Ch	+	-
GU-43	R & ml	Ch & P	+	-
GU-44	R & ml	Ch & P	+	-
GU-45	R & ml	Ch	+	-
GU-51	R & ml	Ch	+	-
GU-52	R & ml	Ch	+	-
GU-53	C	Ch	+	-
GU-54	R & ml	Ch	+	-
GU-62	C	Ch	+	-
GU-64	C	Ch	+	-
GU-65	C	ch	+	-
GU-72	C	Ch & P	+	-
GU-73	C	Ch & P	+	-
GU-75	C	Ch & P	+	-
GU-81	C	Ch & P	+	-
GU-82	C	Ch & P	+	-
GU-93	R & ml	Ch	+	-
GU-103	C	Ch	+	-
GU-104	C	Ch	+	-
GU-105	C	ch	+	-

Annex III: Pictures