ADDIS ABABA UNIVERSITY

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ANTIMICROBIAL ACTIVITY OF LACTIC ACID BACTERIA ISOLATED FROM 'ERGO', ETHIOPIAN TRADITIONAL FERMENTED MILK, ON SOME FOODBORNE PATHOGENS

By

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Declaration

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

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Place and Date of submission November 2006

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LIST OF ABBREVIATIONS

ATCC- American Type Culture Collection

CF-Culture Filtrate

DNA- Deoxy Ribonucleic Acid

E.coli- *Escherchia coli*

LAB- Lactic Acid Bacteria

MIC- Minimum Inhibitory Concentration

MRS- de Mann Rogosa Sharp agar

NORAD- Norwegian Agency for Development Cooperation

rRNA- ribosomal Ribonucleic Acid

S.aures - *Staphylococcus aures*

S.flexineri -*Shigella flexineri*

ssp- Sub species

S.typhi - *Salmonella thyphy*

ABSTRACT

"Ergo" is a traditional Ethiopian fermented milk produced by spontaneous fermentation using traditional utensils under non-hygienic environment. To produce microbiologically safe and chemically defined product; study on the production, processing, utilization and handling of 'Ergo' under different agro-ecological zones as well as the isolation and characterization of potential lactic acid bacteria strains with the potential production of inhibitory factor should be the first essential step. In this study ninety-smallholder farmers from the three districts (Lumme, Fentale, Adami Tulu), thirty from each district, who produced and processed milk were purposeively selected and individually interviewed using a semi-structured questionnaire to asses the production, processing, utilization and handling of traditional fermented milk, "Ergo". Milk fermentation takes relatively longer period (3.57 $\pm$ 1.04 days) in Lumme district than the two districts Fentale and Adami Tulu (1.03$\pm$ 0.183). All households (100%) in Fentale consume un-boiled milk; rather they utilize it in its fermented or raw state, where as only 40% and 46.6% of households in Lumme and Adami Tulu areas, respectively, used fermented milk for consumption. Even though milk is fermented and consumed in all these areas a very limited hygienic care is practiced, 93.4% of the households do not heed for the sanitary aspect of the milk and milk products. To evaluate the antimicrobial activity of the lactic acid bacteria (LAB), 112 strains of LAB, belonging to *Lactococcus, Lactobacillus, Entorococcus, Streptococcus, Leuconostoc* and *Pediococcus* were isolated from Ethiopian traditional fermented milk, "Ergo". Moreover the culture filtrates of all the isolates were examined for antimicrobial activity on some food born pathogens *Salmonella thyphi, Shigella flexineri , Staphylococcus aures* (ATCC-25923) and

Escherchia coli (ATCC-25922) using disc diffusion assay method. Inhibition diameters obtained with the inhibitory substance of 2mm and above inhibition zone over the control were considered as positive. Twelve strains of lactic acid bacteria that produced the greatest antimicrobial substance were selected. These strains were identified as species of *Lactobacillus acidophilus(2)* , *Lactobacillus plantarum(2), Lactococcus lactic spp crimoris(2)* , *Lactococcus lactic spp lactic(3), Leuconostoc lactic* (1) , *Pediococcus pentosaceus(1)* and *Pediococcus sp.(1).* All the inhibitory substance-producing strains were tested for their temperature and pH stability. The antimicrobial activity of the culture filtrate of isolated strains were completely inactivated when treated at 121^{o}C for 15 minutes, where as at 30, 60 and 80^{o}C there were no Significant ($P>0.05$) difference in diameter of inhibition compared to untreated (control). The entire culture filtrate were stable with in a wide range of pH (2-10) and no significant ($P>0.05$) difference was observed between the treated filtrate and the untreated (control). However treating the culture filtrate at pH 12 has significantly ($P<0.05$) decreased the inhibition diameter.

Key words: Lactic acid bacteria, Inhibition, Fermented milk, "Ergo", *E.coli, S. typhi, S. flexineri, S.aureus,* Discdiffusion assay

1. INTRODUCTION

1.1. Food fermentation

Fermentation was one of the ancient methods used by man to produce and preserve foods. Microbial fermentation have played an important role in food processing for thousands of years. Fermentations provide a way to preserve food products, to enhance nutritive value, to destroy undesirable factors, to make a safe product, to improve the appearance and test of some foods, and to reduce the energy required for cooking (Lopez, 1992). These significant changes causing desirable biochemical effects involve in the development of new aroma, flavor, and taste and texture there in increasing the sensory quality, palatability and acceptability of the product. Besides this, fermentation is relatively a low energy preservation that promotes the shelf life of products (Cooke *et al*, 1987).

1.1.1. Traditional food fermentation

Traditional fermentations are those mediated by the hydrolytic influence of indigenous flora or deliberately added enzymes derived from the microbial activity of the substrates. The process employs the entire natural microflora that could function under the varied environmental and non-sterile conditions (Latunda-Dada, 1997).

Traditional methods of preparing fermented foods are not complicated and do not require expensive equipment (Digen, 1982 cited in Negatu, 1992). Fermentation of indigenous foods is, therefore, considered by many to be an effective, inexpensive, and nutritionally

beneficial household technology for communities with food scarcity and malnutrition (Jay, 1994). In traditional LAB fermented foods, it is very common to use and follow controlled natural fermentation processes. This is usually done by ecological control of starter microflora, which is naturally selected as determined by the physico-chemical conditions of caring out of fermentation.

1.1.2. Milk and milk products fermentation and processing in Ethiopia

In Ethiopia, Milk is produced in all the agricultural production systems. The bulk (98%) of the Milk is produced in rural areas by subsistent farmers (Tsehay, 1998). Although milk is produced in almost every production system of Ethiopia, a minor portion of this milk enters the commercial sectors. Farmers close to the main road of Addis Ababa do not have market problems. They can sell their milk directly to consumers or to traders, as well as to the Addis Ababa dairy industry through established milk collective systems, or to the private dairy plants such as Mama Dairy Private Limited Company. Elsewhere in Ethiopia, farmers near towns generally have ready outlets for their liquid milk. However, most farmers live far away from major roads and have no nearby markets and for the fact that milk is relatively perishable food and a high percentage is consumed in a relatively natural state, handling of milk and its products to preserve its natural and desired characteristics is very important (Duane and Cunnignam, 1991).

Because of the generally prevailing unorganized processing, transport and marketing facilities, the people of the tropical countries have mastered the art of conserving or processing the limited quantity of the milk they produce. In small-scale milk production and for those living in rural areas, pasteurization of milk is rarely practiced except

smoking of containers by many rural dairy producers (Taye, 1998). Mogessie and Fekadu, (1993) in their study reported that smoking was found to lower the microbial load as compared to unsmoked containers.

Traditional milk containers are smoked with "Ejersa" (*Olea africana*) splinters together with the leaves into which the raw milk is added every day and let to undergo natural fermentation at ambient temperature. The coagulated (curdled) milk called 'Ergo' and 'Ititu' with their characteristic aromas and flavors would be commonly relished alone, as part of the meal or might also be churned for butter production (Urga, *et al.*, 1992).

1.1.3. "Ergo" Fermentation

The rural people in Ethiopia produce fermented milk by traditional methods. The major fermented milk products produced by smallholder farmers by traditional methods include "Ergo" (fermented sour milk), "Ititu" (Fermented milk curd), "Kibe" (traditional butter), "Neter kibe" (spiced butter), "Ayib" (cottage cheese), "Arerra" (Sour defatted milk), and "Aguat" (whey). "Ergo" is a traditional naturaly 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 Gonfa, 2001). Microorganisms involved in the culturing of "Ergo" were studied by Almaz Gonfa *et.al* 1999 and reported that *Lactococcus garvieae* and *Lactococcus lactis* sub sp.l*actis.* The *Lactobacilli* produced D-Lactic acid and belonged to one spices, but they appeared to be different from other species of the genus *Lactobacillus.*

"Ergo" is mainly produced by women who 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 Fita, 2004). As the major fermented dairy product, "Ergo" is popular and is consumed in all part of the country and by every member of the family. It is known by many different names by many ethnic groups in Ethiopia. "Ergo" is considered as a special food which serves as a basis for further processing and it is particularly used as a nutritional support to sick people, children and to pregnant and lactating mothers of the family, whilst in the lowland pastoral regions fresh milk is preferred (O'Connor, 1994).

"Ergo" fermentation is usually natural, with no defined starter cultures used to initiate it. This is made possible only through the proliferation of the initial milk flora, with microbial succession determined by chemical changes in the fermenting milk. In most urban homes, no attempt seems to be made to control the fermentation. Raw milk is either left at ambient temperatures or kept in warmer places to ferment. In rural areas, particularly among the pastoralists raw milk is usually kept in a well-smoked container and milk from a previous fermentation serves as inoculum. Lactic acid bacteria also become established on the inner walls of the container and serve as starter cultures. Incubation temperature dose not usually vary significantly and the test of the fermented product may, in general, be more or less uniform (Mogessie Ashenafi, 2002).

Almaz Gonfa *et al.* (1999), reported that "Ergo" fermentation is carried out by lactic acid bacteria belonging to the genera *Lactococcus, Streptococcus, Luconostoc*, and *Lactobacillus.* They also observed that *Micrococcus sp*., 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.

1.2. Lactic acid bacteria

Lactic acid bacteria (LAB) comprise a divers group of Gram positive, non-spore forming cocci, cocco-bacili or rods. In most cases they are anaerobic, Microaerophilic or aero-tolerant in their oxygen demands. Although some may produce pseudocatalase when grown at low sugar concentrations, they are generally catalase and oxidase negative. They are chemo-organotrophic and grow in complex media. LAB in general are non-pathogenic to man and animals. They need fermentable carbohydrates for growth and produce lactic acid as a sole or main product from the energy – yielding fermentation of sugars.

1.2.1. Classification

The basic taxonomic unit in bacterial taxonomy is the species and it is a prerequisite to describe and characterize this basic taxonomic unit prior to assigning isolation into a specific taxon. Bacterial classification, however, has remained being determinative for a very long time although there was a need to make it natural. In principle, it is highly likely for bacterial taxonomy to remain stable. Nevertheless, however rigorous an identification method is, because of the dynamic nature of bacterial taxonomy, there has been a lot of instability as more sensitive methods have been developed and consequently new knowledge illuminated additional insights in time. The fundamental reason behind

this fact was that bacteria lack meaningful development stages and phylogenically sound information for fossil records could not be obtained. Therefore, the major break-through on the phylogeny of prokaryotes became obvious after the work by Carl Woese and co-workers in the seventies where they introduce rapid procedures of comparative 16S rRNA sequence analysis and construction of phylogenetic tree (Ludwig and Schleifer, 1994)

The lactic acid bacteria consist of several genera, which include *Streptococcus, Entrococcus, Lactococcus, Luconostoc, Lactobacillu* and *Pediococcus*. Based on similarities in physiology, metabolism and nutritional needs, these genera are grouped together. A primary similarity is that all members produce lactic acid as a major or virtually sole end product of the fermentation of sugars (John, 1998). Species belong to *Streptococcus* and *Leuconostoc* produce the least amount of acid; the homofermentative species of *Lactobacillus* produce the greatest amount of acid. Heterofermentative *Leuconostoc* and *Lactobacillus* species convert glucose to about 50% lactic acid, 25% acetic acid and ethyl alcohol, and 25% carbondioxid. This is important in flavor development and in leavening of certain bread like fermented foods (Jay, 1994).

1.2.2. Role in food preservation

Lactic acid bacteria (LAB) are widely utilized to produce fermented foods contributing to flavor development as well and safe metabolic activities while growing in foods therein utilizing available sugar for the production of organic acids and other metabolites (Nigatu, 1998). In common fermented products such as yogurt, lactic acid is produced by the starter culture bacteria to prevent the growth of undesirable microorganisms

(Daeschel, 1993). Despite improved manufacturing facilities and implementation of effective process control procedures such as Hazard Analysis and Critical Control Point (HACCP) in the food industries, the number of food born illnesses has increased. Nowadays consumers favor food with few chemical preservatives. As a result, there is increased interest in the preservation through LAB because of their safe association with human fermented foods. Several metabolic products produced by these bacteria have antimicrobial effects, including organic acids, fatty acids, hydrogen peroxide and diacetyl. However, attention has focused on the ability of LAB to produce specific proteinaceaus substances, bacteriocins that inhibit the growth of pathogens, such as *Listeria, Clostridium, Staphylococcus, Bacillus spp*. and *Enterococcus spp*., Therefore they enhance the shelf life of the food (Soomro *et al.*, 2002).

1.2.3. Lactic acid bacteria as a probiotics

Food fermentation has a great economic values and it has been accepted that these products contribute in improving human health. Lactic acid bacteria have contributed in increased volume of fermented foods worldwide especially in foods containing probiotics or health promoting bacteria.

Probiotics are live microbial, dietary supplements or food ingredients that have a beneficial effect on the host by influencing the composition and metabolic activity of the flora of the gastrointestinal tract. The concept of ingesting live bacteria as a means of modulating the gut flora to maintain health and promote beneficial effect is not new. At the beginning of the twentieth century, the Nobel Laureate Elie Metchnikooff was the first to propose a scientific rationale for the beneficial effect of the bacteria in yogurt. In his book "*The prolongation of life*" he postulated that yogurt consumption played a role in health and he attributed the longevity of Bulgarian peasants to their intake of yogurt containing *Lactobacillus* species. Tissier in 1906 advocated the administration of *Bifidobacteria* to infants suffering from diarrhea, believed that *Bifidobacteria* displayed the pathogenic bacteria. In Japan, in the early 1930s, Dr. Minoru Shirota focused his research on selecting beneficial strains of lactic acid bacteria (LAB) that could survive passage through the gut and on the use of such strains to develop fermented milk drinks (Saminen and Ouwehand, 2002).

The efficiency of a probiotic strain in producing a given health effect includes at least the following: Adherence to intestinal mucosa and mucus, production of antimicrobial substance, antagonism against pathogens and cariogens, competition for adhesion sites

(Competitive exclusion), interaction with gut-associated lymphoid tissue (immune modulation), inactivation of harmful components with in the intestinal contents (binding of toxin and regulation of metabolic activity of the intestinal microflora), a trophic effect on the intestinal mucosa (e.g. through the production of butyrate) and over all normalization of the intestinal microflora composition and activity (Perdigon *et al*., 1990).

1.2.4. Lactic acid bacteria in Milk fermentation

Lactic acid bacteria (LAB) are widespread in nature and predominate in microflora of milk and its products, many species are involved in the daily manufacturing of dairy products (Ayad *et al.*, 2004).

The lactic acid bacteria used in the dairy fermentation can roughly be divided in to two groups of the basis of their growth optimum. Mesophilic lactic acid bacteria have an optimum growth temperature between 20^{o}C and 30^{o}C and the thermophilic have their optimum between 30^{o}C and 45^{o}C. It is not surprising to discover that traditional fermented products from sub-tropical countries harbor mainly thermophilic lactic acid bacteria, whereas the products with mesophilic bacteria originated from western and northern European countries (Wouters *et al.,* 2002). The thermophilic lactic acid bacteria are best known as a starter for fermented milks. Several varieties of fermented milks originate from countries in Asia Minor and Balkans, like Armenia, Turkeys and Bulgaria. These products have emerged from spontaneous acidification of raw milk by indigenous organisms. Although these organisms have by no means been exhaustively characterized, they consist largely of thermophilic lactic acid bacteria, probably due to the relatively

high incubation temperature determined by the prevailing climate. The first description of milk fermentation by these bacteria can be found in the literature of some hundred years ago (Wauters *et al.*, 2002). Several attempts were made at that time to identify the bacteria dominating the flora in yogurt like products and they were given the names *Bacillus bulgaricus* and *Diplostreptococcus*. These spontaneous fermentation of milk in to yogurt have now been developed in two most frequently used starter bacteria are now classified as *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp *thermophilus*, generally shortened as *Lb.bulgaricus* and *S.thermophilus*, respectively.

The LAB used in commercial starter culture possesses numerous metabolic characteristics such as acidification activity, proteolytic activity, synthesis of bacteroicn, resistance to bacteriophages and production of exopolysaccharaides are strain dependant. All of these important activities contribute to the flavor, texture and frequently the nutritional attributes of the product (Ayad, *et al.*, 2004).

1.3. Antimicrobial substances produced by Lactic acid bacteria

Lactic acid bacteria have an important role in the inhibition of food-born pathogenic and spoilage microorganisms with antimicrobial metabolites, including lactic acid, acetic acid, and other organic acids, hydrogen peroxide, bacteriocins and bacteriocin-like substances (Cadirci and Citak , 2005).

1.3.1. Organic acids

Fermentation by LAB is characterized by the accumulation of organic acids and the accompanying reduction in pH. The levels and types of organic acids produced during the fermentation process depend on the species of organisms, culture composition and growth conditions (Lingren and Dobrogose, 1990).

Lactic acid exerts strong antagonism activity against many microorganisms, including food spoilage organisms and pathogens (ten Brink *et al.*1994). It is the major metabolite of LAB fermentation where it is in equilibrium with its undissociated and dissociated forms, and the extent of the dissociation depends on pH. At low pH, a large amount of lactic acid is in the undissociated form, and it is toxic to many bacteria, fungi and yeasts. However, different microorganisms vary considerably in their sensitivity to lactic acid. At pH 5.0 lactic acid was inhibitory toward spore- forming bacteria but was ineffective against yeast and moulds (Woolford, 1975).

Acetic and propionic acids produced by LAB strains through hetrofermentative pathways, may interact with cell membranes, and cause intracellular acidification and protein denaturation (Huang *et al.*, 1986) They are more antimicrobialy effective than lactic acid due to their higher pKa values (lactic acid 3.08, acetic acid 4.75, and propionic acid 4.87), and higher percent of undissociated acids than lactic acid at a given pH (Earnshaw, 1992).

1.3.2. Hydrogen peroxide and carbondioxide

Hydrogen peroxide is produced by LAB in the presence of oxygen as a result of the action of flavorprotein oxidases or nicotinamide adenine hydroxyl dinucleotide (NADH) peroxidase. The antimicrobial effect of H_2O_2 may result from the oxidation of sulfhydryl groups causing denaturing of a number of enzymes, and from the peroxidation of membrane lipids thus the increased membrane permeability (Kong and Davision, 1980). H_2O_2 may be as a precursor for the production of bactericidal free radical such as superoxide (O_2^-) and hydroxyl (OH^-) radicals which can damage DNA (Byczkowski and Gessner, 1988).

Carbondioxide is mainly produced by heterofermentative lactic acid bacteria. The precise mechanism of its antimicrobial action is still unknown. However, CO_2 may play a role in creating an anaerobic environment, which inhibits enzymatic decarboxylations, and the accumulation of CO_2 in the membrane lipid bilayer may cause a dysfunction in permeability (Eklund, 1984).

1.3.3. Bacteriocins and bacteriocin like substances

Bacteriocins are bacterial peptides that inhibit or kill microorganisms that are usually, but not always, closely related to the producer strain (Cladera-Olivera *et al.*, 2004). Bacteriocins produced by LAB are the subject of intense research because of their antibacterial activity against food-born bacteria. Bacteriocin producing strain of LAB may be very important in competing with other organisms in the intestine. They consist of a biologically active protein moiety, have a bactericidal mode of action and attach to

specific cell receptor. Bacteriocins are heterogeneous group of bacterial antagonists that vary considerably in molecular weight, biochemical property, range of sensitive host and mode of action. Klaenhammer (1993) define the term as protein or protein complexes with bactericidal activity directed against species that are usually closely related to the producer bacterium. Both Gram-negative and Gram-positive bacteria produce them. The Gram-negative bacteriocins are colicin, which are produced by strain of *E.coli* (Braun *et al.*, 1994).

Most of the Gram-positive bacteriocins are membrane active compounds that increase the permeability of the cytoplasmic membrane (Jack *et al.,* 1995). They show much broader spectrum of bactericidal activity than the colicins. Many bacteriocins of LAB are safe and effective natural inhibitors of pathogenic and food spoilage bacteria in various foods. Nisin is the classic example; it prevents C*lostridial* spoilage of processed and natural cheeses, inhibits the growth of some psychrotrophic bacteria in cottage cheeses, extendes the shelf life of milk in warm countries, prevents the growth of spoilage *lactobacilli* in beer and wine fermentations and provides additional protection against *bacillus* and *clostridial* spores in canned foods. Nisin is a permitted food additive in more than 50 countries including the USA and Europe under the trade name Nisaplin (Soomro *et al.*, 2002).

1.4. Methods used for evaluation of antimicrobial activity

Among many methods available for evaluation of antimicrobial activity (Zhennai, 2000), the methods discussed below are commonly used.

1.4.1. The agar diffusion method

The agar diffusion method has long been used for testing antimicrobial activity, and Fleming first used it in 1924 (Piddock, 1990). The method has been widely used for evaluation of antimicrobial activity, especially for biologically derived compounds. It includes agar well diffusion assay and disc assay. In this test, an antimicrobial compound diffuses into agar resulting in concentration gradient that is inversely proportional to the distance from the disc or well is measure the degree of inhibition. The results of the test are generally qualitative (Parish and Davidson, 1993). The method requires that the indicator organisms must grow rapidly, uniformly, and aerobically. Since highly hydrophobic antimicrobial compounds can not diffuse in agar, they are not suitable for test by this method (Piddock, 1990).

Christiansen *et al.* (2005) used agar well diffusion assay to evaluate the antimicrobial activity of the *Lb. paracasei* strains against each other and against other *Lactobacillus* species at 30 ^{o}C on MRS agar. According to Schillinger and Lucke (1989), the well diffusion assay, MRS 0.2 agar inoculated with 0.3 ml of an overnight culture of the indicator strain. Wells of 3mm in diameter were cut into these agar plates, and 0.03 ml of the culture supernatant of the potential producer strain was placed into each well.

The antimicrobial activity of lactic acid bacteria isolated from Borde and Shamita, traditional Ethiopian fermented beverages, on some food born pathogens was determined by the disc diffusion method (Tadese *et al.*, 2005). Sterile filter discs (12mm) were dipped in to the culture broth of lactic acid bacteria incubated for 42 hrs. and placed on solidified Muler-Hinton agar (OXOID) seeded with 12 to 14 hr cultures of test

organisms. The plates were kept at 4ºC for 3 to 4 hrs. to permit diffusion on the assay material, and incubated at 37 ºC for 14 to 16 hrs.

The assay methods used for determination of the antimicrobial activity of different species of LAB were slightly different with respect to the sizes of the well, discs and samples, and the incubation conditions were dependent on the indicator organisms used (Zhennai, 2000). Several modified procedures based on the agar diffusion method have also been used for testing antimicrobial activity of LAB. These procedures include the agar spot test and spot-on-lawn assay.

1.4.2. The agar and broth dilution methods

Agar and broth dilution methods are used as quantitative methods, suited for microorganisms with variable growth rate and for anaerobic, microaerophilic microorganisms (Cintas *et al*., 1995). The results are expressed as Minimum Inhibitory Concentration (MIC), which is the lowest concentration of an antimicrobial that prevents growth of a microorganism after a specific incubation period. In this test, an antimicrobial is serially diluted and a single concentration added to a culture tube or plate added with nonselective broth or melted agar medium, which is then inoculated with test organisms and incubated. The MIC is defined as the lowest concentration at which no growth occurs (absence of turbidity) in a medium following incubation (Parish and Davidson, 1993).

1.4.3. The automated turbidometeric assay

A turbidometeric assay based on automated system determines the effect of a compound on the growth or death kinetics of a microorganism. It provides information concerning the effect of an antimicrobial that may cause a delayed lag phase or reduced growth rate at concentrations below the MIC. Since the bacterial growth is monitored by measuring the turbidity of the broth medium, the method demands that the instrument be highly sensitive. Growth at level below log 5.0 CFU/ml may not be detectable (Davidson and Parish, 1989).

1.5. Antimicrobial activity of lactic acid bacteria isolated from Ethiopian traditional fermented foods

The main advantage of natural fermentation processes is that they are fitting to the rural situation, since they were in fact created by it. Also, the consumer safety of several African fermented foods is improved by Lactic acid fermentation, which creates an environment that is unfavorable to pathogenic Entrobacteriaceae and Bacillaceae.

In Ethiopia some research, have been done on the antimicrobial activity of lactic acid bacteria isolated from traditional Ethiopian fermented foods and beverages by different authors. Idris, (1999) on his work in some microbial and biochemical studies on the fermentation of two traditional condiments, 'Awaze' and 'Datta' reported that entrobacteriacea and coliforms, which were isolated at the initial stage of 'Datta' fermentation were inhibited at 32h. by the antimicrobial activity of dominating lactic acid bacteria. Antimicrobial activity of LAB isolated from two traditional fermented

beverages (Borde and Shameta) on *Staphylococcus aures, Shigela flexinery, Salmonella* spp. and *Escherchia coli* O157:H7 was examined. All isolates, except *Escherchia coli* O157:H7, showed additional 3 to 4 mm of inhibition zone over the control. This was <3 mm for *Escherchia coli* O157:H7. *Lactobacillus* isolates were the most inhibitory to the test strains followed by *Pedicoccus, Streptococcus* and *Leuconostoc* isolates in that order (Tadesse, *et al.,* 2005). From the studies compiled in the thesis work of Nigatu (1998), regarding the microbiological safety of the two Ethiopian widely consumed LAB fermented foods, "Kocho" and "Tef", the dough and freshly baked products were found capable of inactivating asporgenous pathogenic and spoilage bacteria. In their report of study on the fate of *Escherichia coli* O157:H7 during the processing and storage of 'Ergo' and 'Ayib', traditional Ethiopian dairy products, Mekonen Tsegaye and Mogesie Ashenafi (2005) stated that the decrease in the population of the *E.coli* O157:H7 test strain in fermenting "Ergo" could have been due to the antagonistic activities of the LAB through their metabolites and resulting low pH.

Fermented milk "Ergo" plays important role in the diet of low income and the majority of people in the rural areas of Ethiopia using trtaditional utensils. But if "Ergo" is to be produced on large scale some tasks have to be undertaken according to Mogessie Ashenafi (2002), Thus it is logical to start with isolating and characterizing as many lactic acid bacteria as possible from 'Ergo' produced in the various ecological zones of the country. These cultures could be identified, selected and various combinations of them could be used for a controlled fermentation of 'Ergo'. Moreover, isolation and characterization of promising strains of lactic acid bacteria for use as starter culture is the essential first step in the right direction (Fekadu *et al.*, 1998) and use of starter culture

with the potential to produce inhibitory factors would result in the improved safety of fermented products.

Hence, this project was designed with the following objectives.

2. OBJECTIVES

General Objective:

- ❖ To evaluate the antimicrobial activity of lactic acid bacteria isolates from naturally fermented milk, "Ergo", produced in arid and semi-arid areas of East Shoa Zone of Oromiya Regional State, Ethiopia.

Specific objectives:

- To assess the production, processing and utilization of ‘Ergo’ in the Adami Tulu Jidocombolcha, Lumme and Fentale districts.
- To isolate and characterize lactic acid bacteria from ‘Ergo’ samples from the study areas.
- To evaluate the antimicrobial activity of the isolates on some food born pathogens.
- To determine the effect of pH and temperature on the antimicrobial activity of inhibitory substance produced by the lactic acid bacteria.
- To produce base-line data for further study of the LAB isolates.

3. MATERIALS AND METHODS

3.1. The study areas

The study was conducted in three districts of East Shoa Zone of Oromiya (Adami Tulu-Jidocombolcha, Lumme and Fentale) of Oromiya Regional State (Fig 1). The altitude of these areas ranges from 910-2300 meters above sea level (masl) and have arid and semi-arid type of climate with an erratic, unreliable rainfall averaging between 500 and 900 mm per annum (Lemma Fita, 2004). The rainfall is bimodal with the short rains from February to May, and long rains from June to September. The predominant production system in the area is mixed crop livestock farming, except for Fentale where pastoral and agro-pastroral production system predominates.

3.1.1. Lumme district

The district is located about 75 km south of Addis Ababa. It has an altitude ranging from 1500-2250 masl receiving an average annual rainfall of 900 mm (457-1400mm). The minimum and maximum temperature is 18 and 25 ^{o}C respectively (Lemma Fita, 2004)

3.1.2. Adami Tulu Jido-combolcha

The area is located about 167 Km South of Addis Ababa. It is situated at an altitude ranging from 1500-2300 masl. The area receives an average rainfall of 800mm, ranging from (500-1000mm). The minimum and maximum temperature is 10 and 23 ^{o}C respectively.

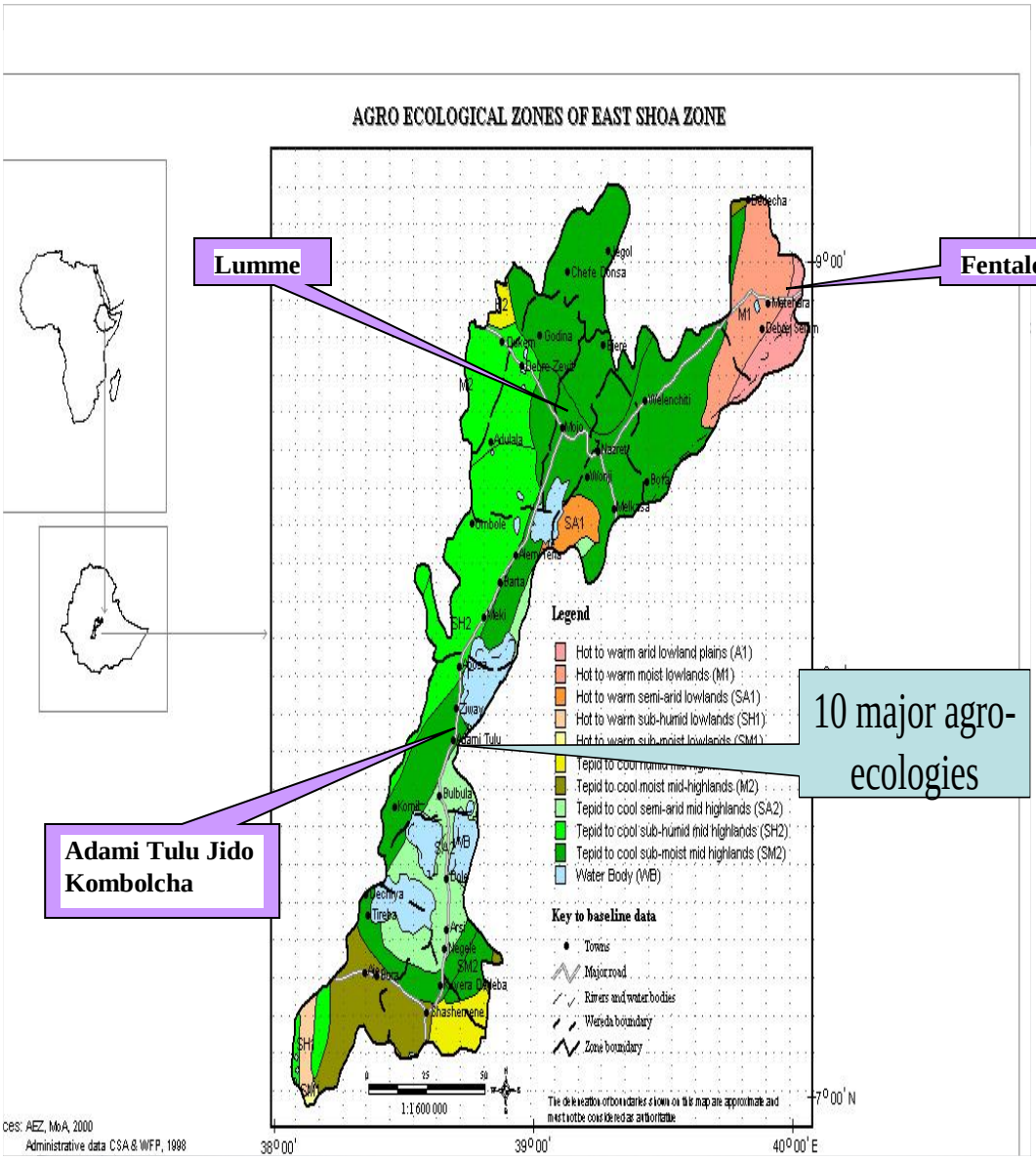

Fig 1. The study districts and the agro-ecological Zones of East Shoa Zone

3.1.3. Fentale district

Fentale is located about 190 Km East of Addis Ababa on Addis-Harer road. It has an altitude of 910-950 masl; with annual rainfall of 560-630mm. Average temperature range is 29-38 ^{o}C. With its arid and semi arid climate pastoral and agro-pastoral production system predominates in the area.

3.2. Data Collection

3.2.1. The Survey

Preliminary survey was conducted in the study areas, Adami Tulu, Lumme and Fentale, to assess production, handling, processing, preservation and consumption of milk and milk products.

Six kebeles from each district were randomly selected. From each Kebele five households who owned milking cows and process milk were purposively selected. Ninety individuals, thirty from each were interviewed using a semi-structured questionnaire (Appendix 1).

3.2.2. The Experiment

3.2.2.1. Test strains

Salmonella thyphi (Clinical isolate), *Shigella flexineri* (Clinical isolate), *Staphylococcus aureus* (ATCC-25923) and *Escherichia coli* (ATCC-25922) were used as test microorganisms. These were obtained from Ethiopian Health and Nutrition Research Institute, Addis Ababa.

3.2.2.2 'Ergo' Sample collection

Samples of 'Ergo' (250 ml) were collected from 10-selected farms from each of three districts, and 30 samples were collected and analyzed. Samples were collected using sterilized flasks and kept under refrigeration temperature using an icebox and brought to the dairy laboratory. Samples were then kept in refrigerator until analysis.

3.2.2.3. Plating, isolation and identification of lactic acid bacteria (LAB)

Plating was done with in 3-6hrs of arrival of the sample in the laboratory. Enumeration of lactic acid bacteria were done after plating well diluted (10^{-5}) 0.1 ml of sample on de Mann Rogosa Sharp agar (MRS) (HIMIEDIA) and incubated anaerobically in anaerobic jar (BBL, GasPak Plus) at 37 ^{o}C for 48hrs. with replications.

MRS medium composition:

Protease peptone	10.00 g/lit
Beef extract	10.00
Yeast extract	5.00
Dextrose	20.00
Polysorbate 80	1.00
Ammonium citrate	2.00
Sodium acetate	5.00
Magnesium sulphate	0.1
Manganese sulphate	0.05
Dipotassium phosphate	2.00
Agar	12.00

Ten colonies were randomly selected from countable MRS agar plate replica. The colonies were purified by successive streaking on appropriate agar media (MRS) before being subjected to characterization. Five colonies with different morphology from each plate were transferred to MRS broth, incubated for about 12 hours and maintained in the refrigerator at 4 ^{o}C . The isolates were grouped as lactic acid bacteria after examining for their Gram reaction, Cell and Colony morphology, Catalase reaction and Gas production from glucose fermentation. Those that are characterized as Lactic acid bacteria were kept as a stock culture in the refrigerator at -20 ^{o}C and in a glycerol solution.

Gram reaction (KOH test):

Two drops of 3% Potassium Hydroxide (KOH) solution were placed on a clean microscope slide. Twenty-four hours old pure culture colony was picked using a sterile wire loop from the MRS agar plate and stirred in the KOH solution for about 2 minutes, and the inoculating loop was then raised slowly from the mass. The Gram-negative ones show the thread of slime followed the loop of 0.5 to 2cm or more, where as the Gram positives do not show slime and the watery suspension.

Cell shape and cell arrangement:

From overnight, pure broth culture smearing on clean microscope slide was employed. The preparation was observed under light microscope using oil immersion objective. The observed cell shapes and cell arrangements were recored.

Catalase test:

A drop of 3% solution of hydrogen peroxide (H_2O_2) were placed on a clean microscope slide, and 24 hour old culture colony from the MRS plate was picked using sterile wire loop and the evolution of gas cause bubbles to form and was indicative of positive test.

Gas Production:

The production of gas during glucose fermentation was observed by placing an inverted Durham tube in MRS broth inoculated with pure culture isolates. After 24hrs of incubation the result were analyzed.

Further grouping in to different genera was made by testing growth at different temperature (10 and 45ºC) and growth at NaCl 4% using Bergey's Manual of Determinative Bacteriology (Holt, 1994) and Herrigan (1998).

3.2.2.4. Determination of pH

pH of culture medias (broth) were determined using a digital 704 pH meter (Metrohm ion analysis, Metrohm ltd.,Herisau,Switzerland) after calibrating using standard buffers (Metrohm ion analysis, Metrohm ltd.,Herisau,Switzerland) at pH 4 and 7.

3.2.2.5. Antimicrobial Activity and Bioassay

The antimicrobial activities of the isolates were quantified by modifying the disc-diffusion method assay procedure of Savadgo *et al.* (2004) and Tadesse *et al.* (2005).

A well-isolated colony was selected from MRS agar plate culture. The top of the colony was touched with a loop, and the growth is transferred into a tube containing sterile 5ml MRS broth. And the broth culture is incubated at 37ºC for about 24hrs. To get the culture filtrate a 24hrs cultures were centrifuged (10,000 rpm for 20min, at 4ºC) then was adjusted to pH 7 by 1M NaOH to exclude antimicrobial effect of organic acid (Savadogo *et al.*, 2004). Two control test materials also were prepared using un-inoculated MRS broth.

An actively growing test (indicator) microorganisms in a Tryptone Soya broth (OXOID)

Pancreatic digest of casein	17.0 g/lit
Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Di-basic potassium phosphate	2.5
Glucose	2.5

of 24hrs culture at 35ºC were dipped with a sterile cotton swab which was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The dried surface of a Mueller-Hinton agar (HIMIDIA).

Beef, infusion from	300.00gm/lit
Casein acid hydrlysate	17.50
Starch	1.50

Plates were inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60ºC each time to ensure an even distribution of inoculum.

A sterile approximately 6 mm in diameter discs (Whatman filter paper no. 1) were delivered with culture filtrate of each isolate by using a wire loop of 20 gauge wire having 2mm of diameter, that can deliver 5µl of the extract to each disc (Lalitha, 2005). Four discs (with culture filtrate) were placed on a 100mm plate with in 5 to 15 minutes of striking of the test organisms.

After 12 and 24 hours of anaerobic incubation, each plate was examined. The diameter of the zones of complete inhibition was measured, including the diameter of the disc. Zones are measured to the nearest whole number in millimeter, using transparent ruler.

3.2.2.6. Characterization and identification of inhibitory substance producing LAB strains

The carbohydrate fermentation profiles of the selected 12 LAB isolates were investigated using API 50 CH strips, and API CHL medium according to manufacturer's instruction (API system, bio-merieux, France). Overnight cultures of the isolates grown in 10ml MRS broth at 30^{o}C were washed twice with sterile peptone water and the pellets were re-suspended in API 50 CHL medium, using sterile pasture pipettes. With subsequent mixing, homogenized suspensions of the cells in the medium where transferred into each of the 50 wells on the API 50 CH strips. Strips were covered as recommended by the manufacturer instruction and incubated at 30^{o}C. Changes in color were monitored after 24hrs of incubation. The first strip served as a control well and Esculine hydrolysis (well 25) was revealed by change to darker or black colors, others changed to yellow or no change at all. Results were represented by positive sign (+) while a negative sign (-) was designated for no change. The LAB strains were tentatively designated to species

according to Bergey's Manual of Determinative Bacteriology (Holt, 1994) in addition to identification table in API 50 CH user manual.

Litmus Milk test:

The inhibitory substance producing isolates were also grown in litmus milk, and fermentation characteristics were recorded. To differentiate bacteria isolates based on various reactions that occur in skim milk supplemented with a litmus pH indicator litmus milk (Skim milk powder 100.00, Litmus 0.50, and Sodium sulphite 0.50 gm/ lit)(Himidia laboratory limited, Mumbai, India) were used.

3.2.2.7. Effect of heat treatment on inhibitory substance produced by LAB isolates from 'Ergo'

The inhibitory substance producing strains were grown in MRS broth for 24hrs, and the culture filtrate was decanted and exposed to various heat treatments

Treatment 1 at 30^{o}C for 30 minutes

Treatment 2 at 60^{o}C for 30 minutes

Treatment 3 at 80^{o}C for 30 minutes,

Treatment 4 at 121^{o}C for 15 minutes

3.2.2.8. Effect of pH on inhibitory substance produced by LAB isolates from 'Ergo'

In order to determine the sensitivity of the culture filtrate recovered during stationary growth phase of the isolates were adjusted to pH levels ranging from 2 – 12 (interval of 2) by using 1M of NaOH and 1M of HCl (Hernadez, *et al*. 2005). After placing in the

incubator for about 4hours, the pH was readjusted at 6.5 and the antimicrobial activity was then determined.

3.3. Statistical analysis

The data collected during the survey were analyzed using descriptive statistics and mean comparison procedure of the Statistical Package for Social Science (SPSS V. 12.0).

All laboratory experiments were repeated three times except the disc diffusion assay, which was done twice. The different sample treatments were compared by performing one-way analysis of variance on the replicates at 95% level of significance using SPSS 12.0 statistical program. Significant results refer to $p<0.05$.

4. RESULTS

4.1. Milk handling, fermentation and utilization in the study area

4.1.1. Factors affecting and duration of milk fermentation

Duration of milk fermentation in Lumme, Adami Tulu and Fentale districts as reported by respondents is given in Table 1.

The time required for fermentation of milk depend on different factors like temperature (climatic condition), the cleaning and smoking practices of the equipment used for fermentation and use of starter or previous fermented milk for back sloping. According to the respondants if the temperature is relatively higher the milk fermented within a short period, while low temperature takes somewhat long period for fermentation.

The duration of milk fermentation is relatively higher in Lumme district (3.57$\pm$ 1.04days) than the two districts Fentale and Adami Tulu (1.03$\pm$ 0.183days) (Table 1). This could be due to the temperature (climatic condition) of the areas.

Fig.2 A women processing (churning) the fermented milk, using a traditional clay pot in Lumme district.

According to respondents, low temperature is the most limiting factor for milk fermentation in Lumme district (96.7%) than smoking and back sloping as well as cleaning the vessels.

Table 1. Duration of milk fermentation in Lumme, Adami Tulu and Fentale districts as reported by respondents.

Districts	Number*	Average in days
Lumme	30	3.57 ± 1. 04
Adami Tulu	30	1.03 ± 0.183
Fentale	30	1.03 ± 0.183

*Number of observation (respondents) in each district

Where as, smoking affects the fermentation time significantly in Fentale (86.7%) followed by Adami Tulu (20%). If the vessels are smoked, fermentation takes longer period for fermenting than in the un-smoked ones (Table 2).

Table 2. Factors affecting milk fermentation time in the three districts by percentage in Lumme, Adami tulu and Fentale districts as reported by respondents.

Factors	Districts		
	Lumme (%)	**Adami Tulu (%)**	**Fentale (%)**
Temperature	96.7 (29)	33.3 (10)*	3.3 (1)
Smoking	0	20 (6)	86.7 (26)
Cleaning vessel	0	6.67 (2)	3.3 (1)
Back sloping	3.3 (1)	26.7 (8)	0
Others	0	13.3 (4)	6.67 (2)

*Numbers in parenthesis indicates the number of observations in each district

4.1.2. Milk production and utilization in the study districts

Average volume of milk production per day per houshold in Adami Tulu district is the highest (6.68 litters) compared to the two districts Fentale (4.10 litters) and Lumme (3.67 litters) (Table 3).

Maximum volume of milk produced per houshold is reported in Lumme (44 litters per day) compared to the other two areas (20 litter per day).

Consumption of raw (unboiled) or unfermented milk is widespread in Fentale and Adami Tulu, with significant health implication.

Table 3. Milk production and utilization in Lumme, Adami Tulu and Fentale districts as reported by respondents.

Districts	Mean (Milk produced/ day/household) (liters)	Utilization (consumption) %		
		Raw	Boiled	Fermented
Lumme	3.67	3.3 (1)*	16.7 (5)	40 (12)
Adami Tulu	6.68	43.3 (13)	26.7 (8)	46.7 (14)
Fentale	4.10	100 (30)	0 (0)	100 (30)

*Numbers in parenthesis indicates the number of observation in each district

4.1.3. Milk and milk products handling

The handling and hygienic practice performed in the study areas (Lumme, Adami Tulu and Fentale) as reported by respondents is given in Table 4. Cleaning the udder and hindquarters before milking is not a common practice in all the study areas. Most of the smallholder farmers (93.4 %) practice limited sanitary procedures before milking.

Only very few farmers (6.6%) practice hand, teat and equipment washing. Milking is done in open-air fields, home shade or under the trees (Fig 3).

However, almost all farmers in the study area practice smoking of milking and fermenting vessels. The most commonly used smoking herb in all the study areas is *Olea africana* "Ejersa" (Fig 4). Moreover, pastoralists in Fentale also use *Balanites aegyptiaca* "Badana"(Fig 5). Some other plants like *Acacia nilotica*, *Heeria reticulala* "Gaarii" are also practiced in the areas by few farmers.

Fig 3. Milking under the tree shade around Adami Tulu Area.

Farmers reported that these plants are used to increase the shelf life of milk and milk products.

Fig 4.*Olea africana* "Ejersa"

Fig 5. *Balanites aegyptiaca* " Badana"

If there is high production of milk, the milking and storage vessels are not smoked perhaps to facilitate fermentation.

Traditional pots that are made from wood, animal skin (mostly in Fentale), clay pots and woven grass baskets are the most common milking and fermenting materials in the three districts. 54.4 % of the respondents in the study areas used traditional milking pot that have a wide opening for milking and 44.4% used plastic pots, however only 1.1% of the respondents used metallic cans. On the other hand, in all the districts traditional pots were exclusively used for fermentation.

Table 4. Milk and Milk products handling and hygiene in Lumme, Adami Tulu and Fentale districts as reported by respondents.

	Lumme	Adami Tulu	Fentale	Total
Equipments used for milking (%)				
1. Plastic	50 (15)	83.3 (25)*	0	44.4 (40)
2. Traditional	50 (15)	13.3 (4)	100 (30)	54.4 (49)
3. Metallic	0	3.3 (1)	0	1.1 (1)
Equipments used for fermentation (%)				
1.Plastic	0	0	0	0
2.Traditional	100 (30)	100 (30)	100 (30)	100 (90)
3.Metallic	0	0	0	0
Hygienic practices				
1.Wash milking equipment, let calf suckle and milk	63.3(19)	73.3 (22)	10 (3)	48.9(44)
2.Wash hand, equipments, the teats and milk	16.7 (5)	3.3(1)	0	6.6(6)
3.Let the calf suckle and milk	20 (6)	23.3(7)	90 (27)	44.4 (40)

*Numbers in parenthesis indicates the number of observation in each district

4.2. Isolation of Lactic acid bacteria

Strains of lactic acid bacteria isolated from fermented milk and their Morphological and biochemical properties are presented in Table 5. Gram positive, Catalase negative, Cocci, Cocco-bacilli or rod shaped isolates with characteristic cell arrangements when grown on

Table 5. Morphological and biochemical characterization of bacteria strains isolated from 'Ergo' that were collected from three districts (Lumme, Adami Tulu, Fentale).

Site	***Gram rxn***		***Catalase***		***Margin***				***Elevation***			***Form***			***Cell shape***			***Cell arrangement***				***LAB identified***						
	+ve	-ve	+ve	-ve	En	Un	Lo	Fi	F	Ri	Co	C	I	R	Co	Cb	Ro	Si	Pa	Ch	Te	Lb	Lc	Lu	Et	Sr	Pd	No
Mj	48	2	18	32	20	16	6	8	27	19	4	19	31	0	28	2	20	12	37	19	0	10	9	0	5	4	2	20
Ad	49	1	2	48	36	4	9	1	8	41	1	37	13	0	26	5	18	3	33	25	1	16	20	3	3	2	4	2
Fn	48	2	16	34	50	0	0	0	2	41	7	47	2	1	39	4	7	17	30	22	0	11	0	5	8	5	5	16
Tot.	**145**	**5**	**36**	**114**	**106**	**20**	**15**	**9**	**37**	**101**	**12**	**103**	**46**	**1**	**93**	**11**	**45**	**32**	**100**	**66**	**1**	**37**	**29**	**8**	**16**	**11**	**11**	**38**

Forms:-Circular (C)
Irregular (I)
Rhizoid (R)

Elevation:-Flat (F)
Raised (Ri)
Convex (Co)

Margin:-Entire (En)
Undulated (Un)
Lobate (Lo)
Filamentous (Fi)

Site: - Lumme (Mj), Fentale (Fn), Adami tulu (Ad)
+ve: - Positive
-ve: -Negative

Lactic acid bacteria (LAB)-
Lb- *Lactobacillus*
Lc- *Lactococcus*
Et- *Entrococcus*
Sr- *Streptococcus*
Pd- *Pediococcus*
Lu- *Luconostoc*
No- None LAB

Cell shape- Cocci (Co)
Coccobacillus (Cb)
Rod (Ro)

Cell arrangment-
Single (Si)
Pair (Pi)
Chain (Ch)
Tetrad (Te)

MRS growth media were considered as lactic acid bacteria (Hernandez, *et al.*, 2005; Savadogo, *et al.*, 2004). From a total of 150 bacteria isolated from the 30 samples (10 from each district) 112 of them are tentatively considered as lactic acid bacteria. Thirty of which are isolated form Lumme, 48 from Adami Tulu and 34 from Fentale districts. The LAB isolated comprised of five genera, *Lactobacillus, Lactococcus, Leuconostoc, Entrococcus* and *Streptococcus*

4.3. Antimicrobial activity of the isolates

The antimicrobial activity of isolated LAB and degree of inhibition is given in Table 6. From a total of 112 lactic acid bacteria isolates that were subjected to antimicrobial activity test on some food born pathogens using disc diffusion method, the extracts of 12 strains of lactic acid bacteria gave zone of inhibition on to indicator pathogenic strain tested. The diameters of inhibition ranged from 7mm to 12mm.

The biggest diameters of 12mm were obtained from the extracts of strains Mj-622 on *Shigella flexinery,* Ad-211 on *Salmonella typhi* and Ad-855 on *Salmonella typhi* and *Shigella flexineri.* In contrast, *Escherichia coli* (ATCC-25922) was the most resistant strain it was only inhibited by two strain (Ad-855 and Fn-133) by a diameter of 10mm and 9mm respectively, followed by *Staphylococcus aures* (ATCC-25923) that was inhibited by four strains with low diameter of inhibition, Strain Ad-211 by the diameter of inhibition 9mm, Ad-233 by 10mm, Ad-411 by 10mm and Ad-522 by 10mm.

Table 6. Average inhibition zone of antimicrobial activity of the inhibitory culture filtrate, on some food born pathogens, Using disc diffusion assay technique.

No	LAB strain	Test bacteria	Diameter of inhibition (mm) (Mean ± SD)
1	Mj-622	*Salmonella typhi*	10 ± 0.00
		Shigella flexineri	11.75 ± 0.354
2	Ad-211	*Salmonella typhi*	11.75 ± 0.354
		Shigella flexineri	10 ± 0.00
		Staphylococcus aureus (ATCC-25923)	9 ± 0.00
3	Ad-233	*Salmonella typhi*	9 ± 0.00
		Shigella flexineri	10 ± 0.00
		Staphylococcus aureus (ATCC-25923)	10 ± 0.354
4	Ad-355	*Salmonella typhi*	9.5 ± 0.707
		Shigella flexineri	9 ± 0.00
		Staphylococcus aureus (ATCC-2592)	9.25 ± 0.354
5	Ad-411	*Salmonella typhi*	10 ± 0.00
6	Ad-522	*Shigella flexineri*	10.5 ± 0.00
		Staphylococcus aureus(ATCC-25923)	9.25 ± 0.354
7	Ad-855	*Salmonella typhi*	11.75 ± 0.354
		Shigella flexineri	11.25 ± 0.354
		Escherichia coli (ATCC-25922)	10 ± 0.00
8	Fn-133	*Salmonella typhi*	10 ± 0.00
		Escherichia coli (ATCC-25922)	9 ± 0.00
9	Fn-144	*Salmonella typhi*	9.25 ± 0.354
10	Fn-233	*Salmonella typhi*	9.25 ± 0.354
11	Fn-533	*Shigella flexneri*	9 ± 0.00
12	Fn-555	*Shigella flexneri*	9 ± 0.00

Table 7. Morphological, cultural and physiological characteristics of inhibitory substance producing strains of lactic acid bacteria isolated from 'Ergo'.

Characteristics	**Inhibitory substance producing lactic acid bacteria isolates**											
	Mj-622	**Ad-211**	**Ad-233**	**Ad-355**	**Ad-411**	**Ad-522**	**Ad-855**	**Fn-133**	**Fn-144**	**Fn-233**	**Fn-533**	**Fn-555**
Cell morphology	Coc	Coc	Coc-baci	Coc	Coc	Rod	Coc	Coc	Coc	Rod	Coc	Coc
Cell arrangement	Pr	Pr,S.ch	Pa,S.ch	Pair	Pr & ch	Pr&ch	Pr&ch	S.ch	Pr	Pr	Pr&ch	Pair
Gram stain reaction	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Spore formation	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Colony morphology	Ft & Irg	Ra & cir	Ra& cir	Ra& cir	Ra &cir	Ra&cir	Ra&cir	Ra&cir	Ra&cir	Ra&cir	Ra&cir	Ra&cir
Catalase activity	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Glucose fermentation.	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Growth at 10	+ve	+ve	-ve	ND	+ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve
Temperature(^{o}C) 45	-ve	+ve	-ve	-ve	+ve	ND	-ve	ND	+ve	ND	-ve	+ve
Growthin medium 4 NaCl (%)	-ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve
Milk curdling	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve
pH optimum	5.20	5.11	4.43	4.41	4.13	4.13	4.41	4.14	4.20	4.13	4.50	4.22

Legend: Positive reaction (+), Negative reaction (-ve), Not Determined (ND), Flat (Ft),Raised (Ra), Circular (Cir), Irregular (Irg), Pair (Pr), Short chain (S.ch), Chain (Ch)

Table 8. The carbohydrate fermentation profile of inhibitory substance producing lactic acid bacteria isolates from 'Ergo, using API 50 CH strips and API CHL medium.

Sugar	**Mj-622**	**Ad211**	**Ad-233**	**Ad-355**	**Ad-411**	**Ad-522**	**Ad-855**	**Fn-133**	**Fn-144**	**Fn-233**	**Fn-533**	**Fn-555**
3-D-Arabinose	-	-	-	-	+	-	-	-	-	-	-	-
4-L-Arabinose	-	-	+	-	+	-	-	-	+	-	ND	-
5-Ribose	**ND**	+	+	+	+	+	+	-	+	-	-	+
6-D-Xylose	-	-	-	-	+	-	-	-	-	-	ND	-
7-L-Xylose	-	-	-	-	-	-	-	-	-	-	+	-
10-Galactose	+	+	+	-	-	+	-	+	+	-	+	+
18-Mannito	-	+	+	+	+	+	-	-	-	+	ND	+
19-Sorbitol	-	-	+	-	-	-	-	-	-	-	-	-
20-α-Methyl-D-Mannoside	-	-	-	+	-	-	-	-	-	-	-	-
22-N-Acetyl-Glucosamine	+	+	+	-	+	+	+	+	ND	+	+	+
23-Amygdalin	-	+	+	-	+	+	-	+	ND	-	-	+
24-Arbutin	-	+	+	-	+	+	+	+	+	-	ND	+
25-Esculin	+	+	+	+	+	+	+	+	+	+	-	+
26-Salicin	-	ND	+	-	+	+	+	+	+	+	+	+
27-Celiobiose	-	+	+	-	+	+	+	+	+	+	+	+

28-Maltose	-	+	+	+	+	+	-	+	+	+	+	+
29-Lactose	+	+	+	+	+	+	+	+	+	+	+	+
30-Melibiose	-	-	+	-	-	-	-	-	ND	-	+	+
31-Sucrose	+	+	+	-	+	+	+	+	+	+	+	+
32-Trehalose	+	+	+	+	+	-	+	+	+	+	+	+
33-Inulin	_	-	-	+	-	-	-	-	-	-	-	-
34-Melezitose	-	-	+	-	-	-	-	-	-	-	-	-
35-Rafinose	-	-	-	-	-	-	-	-	+	-	-	-
39-Gentiobiose	-	+	+	-	+	+	+	+	+	-	-	+
42- D Tagatose	-	-	-	-	+	-	-	-	--	-	-	-
46- L Arabitol	-	-	-	+	-	-	-	-	-	-	-	-

Not determined (ND), Positive (+), Negative (-)

All the isolates were negative (-) for Glycerol, Erythritol , Adonitol, β-Methyl D-xyloside, Sorbose, Rhamnose, Dulcitol, Inositol, α-Methyl-D-Glucoside, Melezitose, Starch, Glycogen, D-Turanose, D-Lyxose, D-Fucose, L-Fucose, D-Arabitol, Gluconate, 2-Keto Gluconate, 5-Keto Gluconate. And positive for Glucose, Fructose and Mano

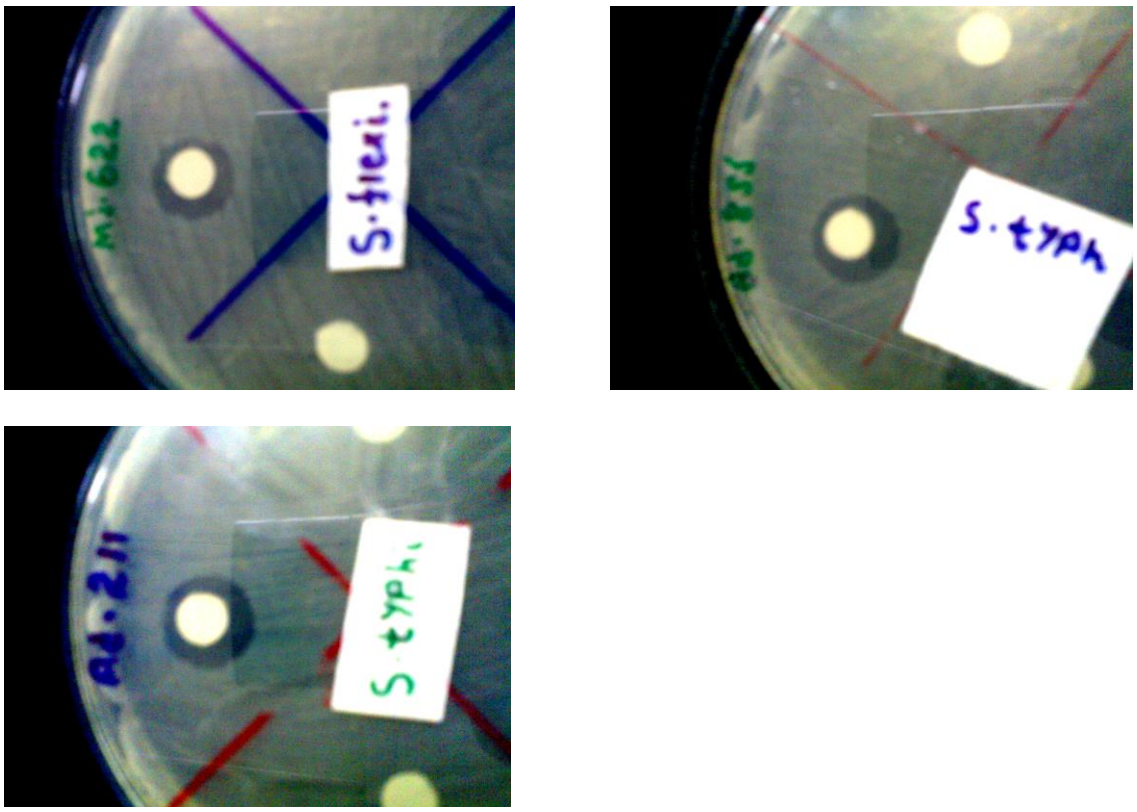

Fig 6. Inhibition Zone on food born pathogens formed by lactic acid bacteria isolated from "Ergo".

4.4. Identification of the inhibitory substance producing strains

The morphological and physiological characteristics, and the carbohydrate fermentation profile of the inhibitory substance producing LAB isolates are given in table Table 7 and Table 8.

Twelve strains among one hundred and twelve lactic acid bacteria isolated from "Ergo" were selected according to their maximum antimicrobial activity against indicator strains. Two of these isolates were identified as *Lactobacillus plantarum* 2 (Ad-522 and Ad-233), two as *Lactococcus lactic* ssp *cremoris* of different strain 1 and 2 (Mj-622 and Ad-855 respectively) three as *Lactococcus lactic* ssp *lactic* of different strains (Fn-555 and Ad-411 as strain 1 and

Ad-211 as strain 2), two as *Lactobacillus acidophyilus* of different strain 1 and 3 (Fn-133 and Fn-233 respectively), one as *Leuconostoc lactic* (Fn-533), one as *Pediococcus pentosaceus* 2 (Fn-144) and the remaining one isolate was not possible to identify to species level, therefore it (Ad-355) was named as *Pediococcus sp*.

4.5. Effect of temperature treatment on the inhibitory activity of the culture filtrate

The antimicrobial substances produced by the isolates were relatively stable during heat treatments at 30 ^{o}C, 60 ^{o}C and 80 ^{o}C for 30 minutes (Fig 7). Treating the culture filtrate at 30 ^{o}C for 30 minutes showed no difference from the control (un treated), as well as heat treating at 60 ^{o}C and 80 ^{o}C for 30 minutes also did not show significant difference ($P>0.05$) from the control. However sterilization (121^{o}C for 15 minutes) led to complete inactivation of the inhibitory activity of the extract, and showed significant difference ($P<0.05$) from the control.

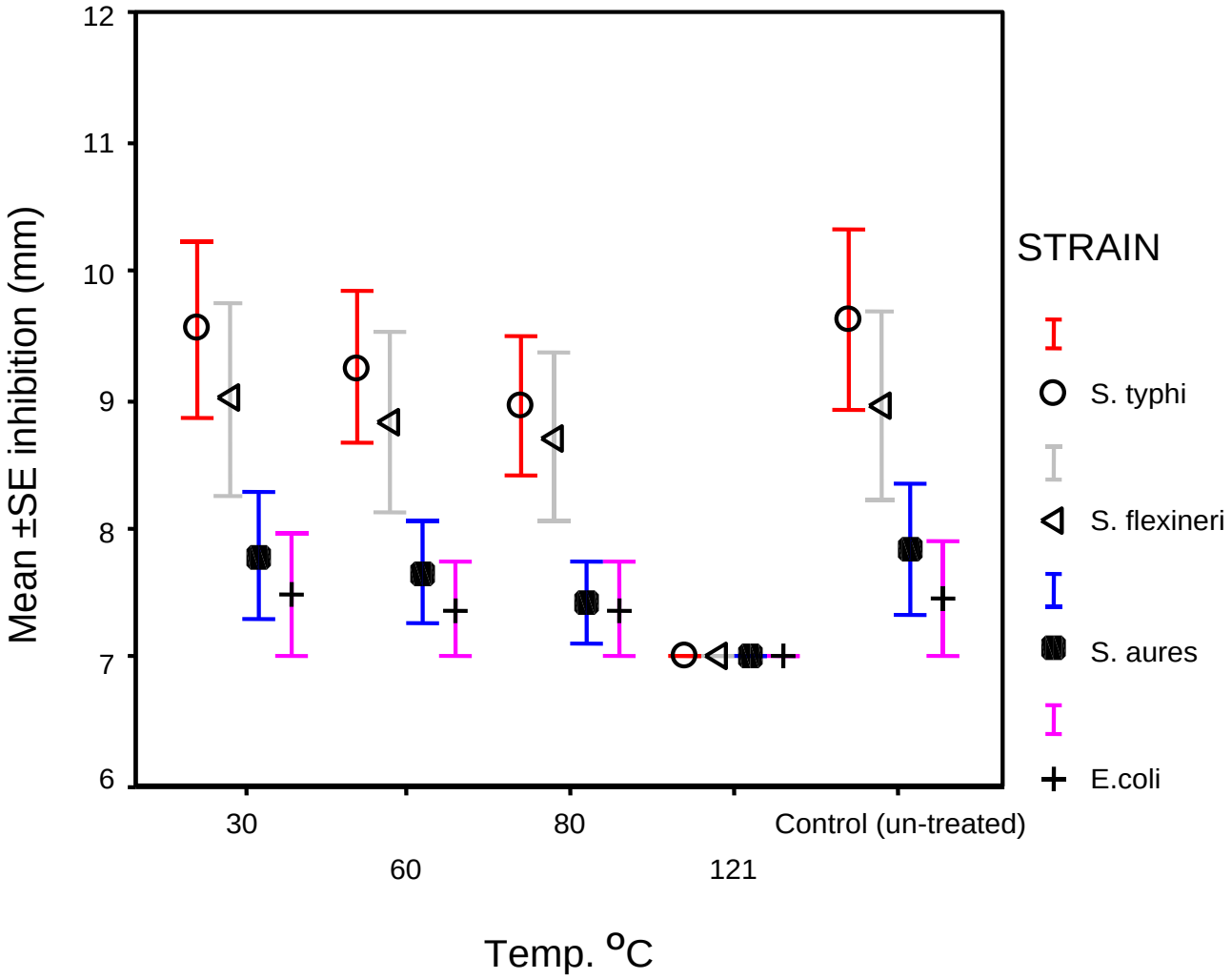

Fig 7. Effect of temperature on antimicrobial activity of inhibitory substance produced by lactic acid bacteria strains isolated from 'Ergo'

4.6. Effect of pH on the inhibitory activity of the culture filtrate

The antimicrobial activity of the culture filtrate after treatment at different pH range (2-10) was not significantly ($P>0.05$) affected (Fig 8). But the antimicrobial activity of the culture filtrate was significantly ($P<0.05$) affected and thus decreased at pH of 12.

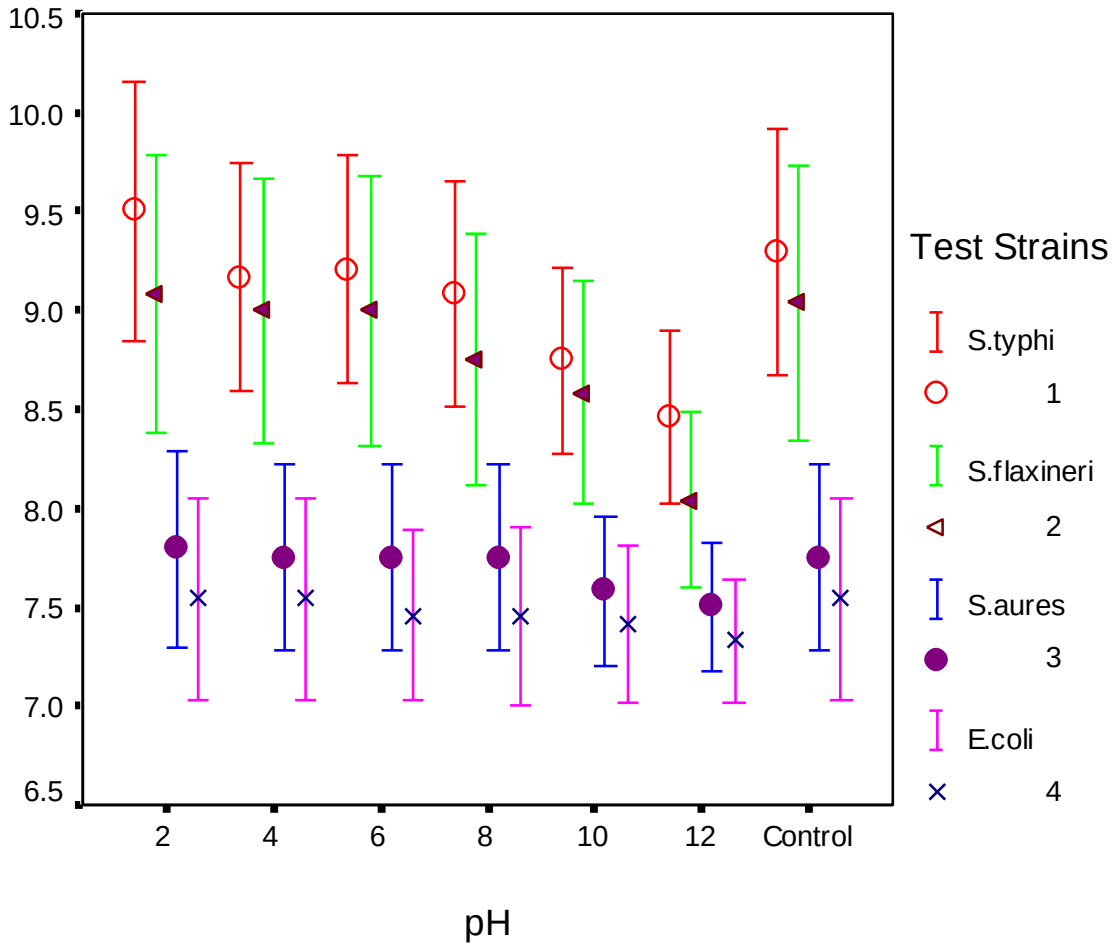

Fig 8. Effect of pH on antimicrobial activity of inhibitory substance produced by lactic acid bacteria strains isolated from 'Ergo'.

5. DISCUSSION

From the preliminary survey conducted in the study areas, small-scale production and processing of milk with poor handling and hygienic control was observed. In most cases no attempt is made to wash the udder before milking. This may be due to the practice of allowing the calf to suckle before milking. Among commonly observed bad milking practices is that of the milker dipping his/her fingers in to the milking vessel as means of lubricating the teats during milking, this was also reported by Kurwijila (1989) in most parts of Southern and East African countries.

In all the study areas milking is practiced in unhygienic way and unclean environment. O'Connor, (1993) and O'Mahony, (1988) also reported that there are uniform traditional techniques used in different parts of Ethiopia to prepare fermented milk and its products. The milking is done in the shade, kraal, grazing field in front of the house shade, under the trees, none of which are clean environments for milking. In most of the cases the milking animals are also kept with rest of the stock in a shade or in the enclosure during night. These places are usually not kept clean and milking cows become soiled with dung and urine. Milk could be contaminated during milking operation, as it is not a common practice to clean the udder and hindquarters before milking. The milking and fermenting vessels are thoroughly washed before smoking treatment. However Bekele (1989) considers the cleanliness of the washing water to be doubtful. The hot embers placed in the vessels for a few minutes bring about the disinfecting effect of the smoking treatment. Smoking of the milking and storage vessels is frequently made in order to extend the fermentation to accumulate the desired volume of milk for churning. This agrees with the finding of Mogessie and Fekadu (1993), Lemma Fita (2004), Kurwijila (1989) that smoking reduced the undesirable microbial contamination that enhance the rate of fermentation and passing the smoke flavour to the milk or milk products. ‘‘Ejersa’’ (*Olea Africana)* is the most commonly used smoking plant in all the

study areas. In other parts of the country such as Eastern Wollega (Alganesh, 2002), and Borena (Bekele and Kassaye, 1987) *Olea africana* was also the most dominant plant used for smoking of milking equipment.

In most cases, the traditional vessels used for milking, storage/fermentation and processing are different. Generally some are made from woven grass, wood fibre, and hollowed wood or skin and clay pot and commonly used for fermentation or processing. These milking and storage equipment are locally called "Qodaa" which were woven from grass and gourd locally termed as ‘‘Migira’’ and "Buqqee" respectively and decorated with seashells and have various sizes. Some of these are known as " Xuunxoo", "Ciicoo","Guchuma", "Elemituu", "Orooboo" and "Baanree" according to their increasing size (Lemma Fita, 2004).

The lactic acid bacteria isolate from fermented milk includes in the *Lactobacillus, Lactococcus, Pediococcus, Entrococcus* and *Streptococcus* genera. These results showed the heterogeneousness of fermented milk (‘Ergo’) at different location or agro climatic zones either in its chemical or microbial composition and quality attributes. These finding was inline with the observations of others who worked on fermented milk, Almaz Gonfa *et al.* (1999) in Ethiopia, Savadogo *et al.* (2004) in fermented milk of Burkina Faso, Ayad *et al.* (2004) in Egypt.

The variation in the type and number of the lactic acid bacteria present as well as the traditional practices (like smoking) would influence the hygienic quality and organoleptic of the fermented milk. Traditionally different herbs and pepper are used in the consumption of fermented milk as beverage, or side dish, perhaps to get acceptable flavor. The variations in the characteristics, quality and acceptability of traditional fermented milk products are the result of unregulated nature of natural fermentation. Indeed some of these organisms could perhaps have beneficial effect on health of the consumers.

All the lactic acid bacteria were subjected to inhibitory activity test using a disc diffusion method. Only twelve isolates (three strains of *Lactococcus lactic spp lactic*, two *Lactobacillus acidophilus*, two *Lactococcus lactic ssp crimoris*, two *Lactobacillus plantarum*, one *Leuconostoc lactic*, one *Pediococcus pentosaceus* and one as *Pediococcus sp*.) showed inhibition zone on some pathogenic bacteria, *Salmonella thyphi* (Clinical isolate), *Shigella flexineri* (Clinical isolate), *Staphylococcus aureus* (ATCC-25923) and *Escherchia coli* (ATCC-25922), to varying degree. Similarly Tadesse *et al*., (2005) and Cadirici and Citak (2005) observed varying degree of inhibition of various food born pathogens by the culture filtrate of lactic acid bacteria, although these inhibitory substances produced by the lactic acid bacteria strains acts differently on the pathogenic reference indicator strains. Inhibitive substances produced by the lactic acid bacteria can be generally protein (Vandenberg, 1993). Inhibition caused by hydrogen peroxide and organic acids was ruled out as the producer strains were cultured anaerobically and the culture supernatant was neutralized before assaying the antimicrobial activity. However, the importance of the inhibition effect varies according to serotypes (Savadoge *et al*., 2004).

The most inhibited indicator strains are the most part of Gram-positive bacteria *Salmonella thyphy* was the most sensitive pathogenic indicator strain to the inhibitory substance produced by the lactic acid bacteria isolates followed by *Shigella flexineri* and *Staphylococcus aureus* (ATCC-25923). Similar results were reported by Savadago *et al.,* (2004) and Tadesse *et al*., (2005). *E.coli* strains were the least sensitive to inhibitory substance produced by the lactic acid bacteria as compared to the other indicator strains. The resistance of Gram negative bacteria is attributed to the particular nature of their cellular envelop, the mechanisms of action described for bacteriocin bringing in phenomenon of adsorption. According to Bhunia *et al*., (1991) the pediocin (bacteriocin produced by *Pediococcus acidilactic*) interact with lipoteichoic acids that are absent in Gram-negative

bacteria. Also all the *Lactococcus lactic* ssp *lactis* isolates (Fn-555, Ad-411 and Ad-211) were not shown inhibitory effect on *E.coli* ATCC-25922. This could be due to the fact that these isolates are nisin producers (Soomro *et al*., 2002) and the primary target of nisin's antimicrobial action is the cell membrane. Nisin has an inhibitory effect against a wide variety of Gram-positive food-born pathogens and spoilage microorganisms (Rodriguz, 1996). The use of nisin in its free form in cheese can be expensive and results in inhibitory effect against the suitable acidification (Robert *et al*., 1992). An alternative to the addition of free nisin to fermented food systems is the use of nisin producing strains during fermentation processes.

All the inhibitory cell free filters of the isolated strains of lactic acid bacteria were completely inactivated when treated at 121^{o}C for 15 minutes. These results are similar with the result by Hernadez *et al.* (2005) in which the antimicrobial substance produced by *Lactobacillus plantarum* TF711 was completely inactivated when treated at 121^{o}C for 15 minutes. However all the culture filtrate were heat stable up to 80^{o}C in addition they all were stable within a wide pH (2-10) range. Heat stability and stability within a wide range of pH was a common characteristic of a number of bacteriocins (Hernadez, *et al*. 2005; Messi *et al.* 2001)

6. CONCLUSION

Despite the fact that ' 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. Training and extension work in dairy product processing and handling is essential. The culture filtrates from twelve strains of lactic acid bacteria isolated from 'Ergo' exhibited antimicrobial activity against four pathogenic test strains. The potential application of the antimicrobial substances as consumer friendly bio-preservatives either in the form of protective culture or as additives is significant besides being less potentially toxic or carcinogenic than current antimicrobial agent. Lactic acid bacteria and their by-products have been shown to be more effective and flexible in several applications. Most inhibitory substances produced by lactic acid bacteria are safe and effective natural inhibitors of pathogenic and food spoilage bacteria in various foods. Due to the properties, such as broad spectrum, heat stability and stability over a wide range of pH, all the inhibitory substances produced by the isolated strains can effectively be used as a bio-preservative in food with a wide range of pH, even after pasteurisation. However a simple in-vitro antimicrobial evaluation of the isolates only can not led to a final conclusion to use the potential strains as a bio-preservative. Additional technological characteristics of the isolates that contribute to the flavour, texture and nutritional attributes of the product such as acidification activity, proteolytic activity, resistance to bacteriophage and production of exopolysacharid should be studied. All the taxonomic determination of the lactic acid bacteria isolates were done by using morphological observations and biochemical tests, so molecular method of identification need to be performed. Further studies can also focus on the characterization of amino acid and nucleotide sequences of these antimicrobial compounds in addition to evaluation of the promising isolates for their probiotic usage.

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Appendices

Apendix I

Evaluation of antimicrobial activity of lactic acid bacteria isolated from 'Ergo', Ethiopian traditional fermented milk.

Questionnair ID_____

Diagonstic survey on the production, handling, preservation and utilization of milk and milk proucts in three districts of East Shewa.

I-General

Date_____

District_____

Name of respondent_____

1. Sex 1- Male 2- Female

2. Age_____

3. Keblea_____

II. Milk and Milk products production

4. How many milking cows do you have? _____

5. Do you have crossbred cows? 1- Yes 2- NO

6. If yes number_____

7. Which cows do you prefer? 1- Local 2-Crossbred

8. Why? _____

9. How many liters of milk (per day) do you get from a cow?_____

10. Is the daily milk production of your cow's optimum? 1- Yes 2- No

11. How do you consume (utilize) your daily produced milk?

1. Raw 1-Yes 2-No

2. Boiled 1-Yes 2-No

3. Fermented 1-Yes 2-No

4. Fermented and processed 1-Yes 2-No

12. What are the products you produce from milk._____?

III. Milk and milk products handling, processing and preservation

13. What kind of handling practices do you use before milking?

1- Wash milking equipments, let calf suckle and milk.

2. Wash hands, the teats and milking equipment, smoke milking equipment and milk.

3. Let the calf suckle and milk

14. What kind of equipment do you use for milking?_____

15. What kind of equipment do you use for storage/ fermentation?_____

16. Do you use any herbs/ plants/ chemicals to facilitate fermentation? 1-Yes 2-No

17. If yes mention the type of herbs/plants/chemicals used and describe how

(application)?_____

18. Mention the herbs/plants used for:

1. Cleaning the milking and fermenting equipments_____

2. Smoking the equipments_____

19.Why do you use these plants?

1- Give good flavor and aroma

2-Increase the shelf life of the milk

3- Facilitate fermentation

4-It just a tradition

5- Other (Specify)_____

20. Why do you process the milk?

1-Surplus production

2-Increase the shelf life

3-For consumption at home

4- For market

5-Other (Specify) _____

21. How many days to ferment milk? _____

22. What factors affect the fermentation time (length)? _____

23. Do you use any preservative to increase the shelf life of milk? 1-Yes 2-No

24. If yes mention the name of the preservative and how it is used?_____

25. For what purpose you use 'Ergo'.

1- For consumption

2-For sale

3-For animal

4-Preserve for further processing

IV. Milk and milk products consumption

26. What percent of the daily milk produced consumed by family members?_____

27. The estimated daily milk, fermented milk 'Ergo', butter 'Kibe'and butter milk 'Areera'consumption of the family?

No	Family member	Milk	Fermented milk	Areera
1	Head of the houseold			
2	The wife			
3	The boy			
4	The girl			
5	Elders (if any)			
6	Others			

28. Do you recall any case of illness related to consumption of milk or other dairy products?

1-Yes 2-No

29.If yes describe it._____

30. What do you think the cause, and how do you think this could be avoided in the future?_____

31. Do you use milk or milk products as a medical treatment? 1- Yes 2- No

32. If yes for which type of disease, its way of application and the particular milk product used?_____

Apendix 2

Morphological and biochemical characterization of bacteria strains isolated from 'Ergo' that were collected from three districts (Lumme, Adami Tulu, Fentale).

	Iso.No	Colonies Morphology			Gram test	Catalase	Cell shape	Cell arrangement.	Gas production.
		Mar	Elev	Forms					
1.	Mj-111	En	Ri	C	+ve	+ve	Cocci	Singel	-ve
2.	Mj-112	En	Ri	C	+ve	+ve	Cocci	Singel	-ve
3.	Mj-121	Lo	F	I	+ve	-ve	Cocci	Pair& chain	-ve
4.	Mj-122	Fi	F	I	+ve	-ve	Cocci	Pair& chain	-ve
5.	Mj-133	Fi	F	I	-ve	-ve	Cocci	Pair	-ve
6.	Mj-211	Lo	F	I	+ve	-ve	Coc-bac	Pair	-ve
7.	Mj-212	Lo	F	I	+ve	-ve	Rod	Single& pair	-ve
8.	Mj-221	En	Ri	C	+ve	-ve	Rod	Pair	-ve
9.	Mj-222	En	Ri	C	+ve	-ve	Rod	Single& chain	-ve
10.	Mj-233	En	Ri	C	+ve	+ve	Cocci	Pair	-ve
11.	Mj-311	Fi	F	I	+ve	-ve	Rod	pair	-ve
12.	Mj-312	Lo	F	C	+ve	-ve	Rod	pair	-ve
13.	Mj-321	En	Ri	C	+ve	+ve	Cocci	Pair& chain	+ve
14.	Mj-322	En	Ri	C	+ve	+ve	Cocci	Pair& chain	-ve
15.	Mj-333	En	Ri	C	+ve	+ve	Cocci	Chain	-ve
16.	Mj-411	Fi	F	I	+ve	-ve	Rod	Pair& chain	-ve
17.	Mj-412	Fi	F	I	+ve	-ve	Rod	Pair& chain	-ve
18.	Mj-421	En	Ri	C	+ve	+ve	Cocci	Tetrad	-ve
19.	Mj-422	Lo	F	I	+ve	+ve	Cocci	Single& pair	-ve
20.	Mj-433	En	Ri	C	+ve	+ve	Cocci	Pair	-ve
21.	Mj-511	Fi	F	I	+ve	-ve	Cocci	Pair	-ve
22.	Mj-512	Fi	F	I	+ve	-ve	Rod	Pair	-ve
23.	Mj-521	Fi	F	I	+ve	-ve	Rod	Pair	-ve
24.	Mj-522	Lo	F	I	+ve	-ve	Rod	Single	-ve
25.	Mj-533	En	Ri	C	+ve	+ve	Cocci	Single	-ve
26.	Mj-611	Un	F	I	+ve	-ve	Cocci	Pair	-ve
27.	Mj-612	Un	F	I	+ve	+ve	Cocci	Pair	-ve

28.	Mj-621	Un	F	I	+ve	-ve	Rod	Pair	-ve
29.	Mj-622	Un	F	I	+ve	-ve	Cocci	Pair	-ve
30.	Mj-633	En	Ri	C	+ve	+ve	Cocci	Pair & chain	+ve
31.	Mj-711	En	Ri	C	+ve	-ve	Cocci	Pair &chain	-ve
32.	Mj-712	En	Ri	C	+ve	-ve	Coco-baci	Pair &chain	-ve
33.	Mj-721	Un	Ri	I	+ve	-ve	Cocci	Pair	-ve
34.	Mj-722	Un	F	I	+ve	-ve	Rod	Pair	-ve
35.	Mj-733	Un	F	I	-ve	-ve	Cocci	Pair	+ve
36.	Mj-811	En	F	C	+ve	-ve	Cocci	Pair& chin	-ve
37.	Mj-812	En	Ri	C	+ve	-ve	Cocci	Pair& chain	-ve
38.	Mj-821	En	Ri	C	+ve	-ve	Cocci	Pair & chain	-ve
39.	Mj-822	Un	F	I	+ve	-ve	Cocci	Single &chain	-ve
40.	Mj-833	Un	F	I	+ve	+ve	Cocci	Pair &chain	
41.	Mj-911	En	Ri	I	+ve	-ve	Rod	Pair	-ve
42.	Mj-912	En	Ri	C	+ve	-ve	Rod	Pair	-ve
43.	Mj-921	Un	F	I	+ve	-ve	Rod	Pair	-ve
44.	Mj-922	Un	F	I	+ve	-ve	Rod	Pair & chain	-ve
45.	Mj-933	Un	F	I	+ve	+ve	Cooci	Chain	-ve
46.	Mj-1011	Un	Co	I	+ve	+ve	Oval	Single	+ve
47.	Mj-1012	Un	F	I	-ve	-ve	Cooci	Pair & short chain	+ve
48.	Mj-1021	Un	Co	I	+ve	+ve	Oval	Single	+ve
49.	Mj-1022	Un	Co	I	+ve	+ve	Oval	Single	+ve
50.	Mj-1033	Un	Co	I	+ve	+ve	Oval	Single	-ve
51.	Ad-111	En	Ri	C	+ve	-ve	Cocci	Tetrad	-ve
52.	Ad-122	En	Ri	C	+ve	-ve	Rod	Pair & chain	-ve
53.	Ad-133	Lo	Ri	I	+ve	-ve	Rod	Pair & chain	-ve
54.	Ad-144	En	Ri	C	+ve	-ve	Cocci	Pair & chain	-ve
55.	Ad-155	En	Ri	C	+ve	-ve	Rod	Single & chain	-ve
56.	Ad-211	En	Ri	C	+ve	-ve	Cocci	Pair & chain	-ve
57.	Ad-222	En	Ri	C	+ve	-ve	Cocci	Pair	-ve
58.	Ad-233	En	Ri	C	+ve	-ve	Coc-bac	Pair & chain	-ve
59.	Ad-244	En	Ri	C	+ve	-ve	Cocci	Pair & chain	-ve
60.	Ad-255	En	Ri	C	+ve	-ve	Cocci	Pair	-ve
61.	Ad-311	Fi	F	Ir	+ve	-ve	Cocci	Pair	-ve

62.	Ad-322	En	Ri	C	+ve	-ve	Rod	Pair & chain	-ve
63.	Ad-333	En	Ri	C	+ve	-ve	Coc-bac	Pair	-ve
64.	Ad-344	Un	Co	I	+ve	+ve	Cocci	Pair & chain	+ve
65.	Ad-355	En	Ri	C	+ve	-ve	Cocci	Pair	-ve
66.	Ad-411	En	Ri	C	+ve	-ve	Cocci	Pair & chain	-ve
67.	Ad-422	Un	Ri	I	+ve	-ve	Rod	Pair	-ve
68.	Ad-433	En	Ri	C	-ve	+ve	cocci	Sing & pair	-ve
69.	Ad-444	Un	Ri	I	+ve	-ve	Rod	Single	-ve
70.	Ad-455	En	Ri	C	+ve	-ve	Rod	Pair	-ve
71.	Ad-511	Lo	Ri	C	+ve	-ve	Rod	Pair & chain	-ve
72.	Ad-522	En	Ri	C	+ve	-ve	Rod	Pair	-ve
73.	Ad-533	En	Ri	C	+ve	-ve	Rod	Pair	+ve
74.	Ad-544	En	Ri	C	+ve	-ve	Rod	Pair	-ve
75.	Ad-555	En	Ri	C	+ve	-ve	Rod	Pair	+ve
76.	Ad-611	En	Ri	C	+ve	-ve	Coc-bac	Pair	-ve
77.	Ad-622	Lo	F	I	+ve	-ve	Rod	Pair	-ve
78.	Ad-633	En	Ri	C	+ve	-ve	Rod	Pair	-ve
79.	Ad-644	En	Ri	C	+ve	-ve	Cocci	Pair & Chain	-ve
80.	Ad-655	Un	Ri	I	+ve	-ve	Rod	Pair	-ve
81.	Ad-711	Lo	F	I	+ve	-ve	Coc-bac	Pair	-ve
82.	Ad-722	En	Ri	C	+ve	-ve	Cocci	Short chain	-ve
83.	Ad-733	En	Ri	C	+ve	-ve	Cocci	Short chain	-ve
84.	Ad-744	Lo	F	I	+ve	-ve	Cocci	Pair	-ve
85.	Ad-755	En	Ri	C	+ve	-ve	Cocci	Short chain	-ve
86.	Ad-811	En	Ri	C	+ve	-ve	Cocci	Short chain	-ve
87.	Ad-822	En	Ri	C	+ve	-ve	Coc-bac	Pair	-ve
88.	Ad-833	En	Ri	C	+ve	-ve	Rod	Pair	-ve
89.	Ad-844	En	Ri	C	+ve	-ve	Cocci	Short chain	-ve
90.	Ad-855	En	Ri	C	+ve	-ve	Cocci	Pair &short chain	-ve
91.	Ad-911	Lo	F	I	+ve	-ve	Cocci	Short chain	-ve
92.	Ad-922	Lo	F	I	+ve	-ve	Cocci	Short chain	-ve
93.	Ad-933	En	Ri	C	+ve	-ve	Cocci	Pair & chain	-ve
94.	Ad-944	En	Ri	C	+ve	-ve	Cocci	Short chain	-ve
95.	Ad-955	Lo	F	I	+ve	-ve	Ovoid	Pair & chain	-ve

96.	Ad-1011	En	Ri	C	+ve	-ve	Cocci	Pair	-ve
97.	Ad-1022	Lo	F	I	+ve	-ve	Ovoid	Pair & Chain	-ve
98.	Ad-1033	En	RI	C	+ve	-ve	Cocci	Short chain	-ve
99.	Ad-1044	En	Ri	C	+ve	-ve	Branching tree like		+ve
100	Ad-1055	En	Ri	C	+ve	-ve	cocci	Pair	+ve
101	Fn-111	En	Co	C	+ve	+ve	Oval	Single	-ve
102	Fn-122	En	Ri	C	+ve	-ve	Cocci	Short chain	-ve
103	Fn-133	En	Ri	C	+ve	-ve	Cocci	Short chain	-ve
104	Fn-144	En	Ri	C	+ve	-ve	Cocci	Pair	-ve
105	Fn-155	En	Ri	C	+ve	-ve	Cocci	Pair & Chain	-ve
106	Fn-211	En	Ri	C	+ve	-ve	Cocci	Single & pair	-ve
107	Fn-222	En	F	I	+ve	+ve	Cocci	Single & pair	-ve
108	Fn-233	En	Ri	C	+ve	-ve	Bacili	Single	-ve
109	Fn-244	En	Ri	C	+ve	-ve	Cocci	Single & pair	-ve
110	Fn-255	En	Ri	C	+ve	-ve	Cocci	Single & pair	-ve
111	Fn-311	En	Ri	C	+ve	+ve	Oval	Single	-ve
112	Fn-322	En	Ri	C	+ve	-ve	Oval	Single	-ve
113	Fn-333	En	Ri	C	+ve	-ve	Cocci	Chain	-ve
114	Fn-344	En	Ri	C	+ve	-ve	Cocci	Long Chain	-ve
115	Fn-355	En	Ri	C	+ve	-ve	Oval	Single	-ve
116	Fn-411	En	Co	C	+ve	+ve	Cocci	Single & short chain	+ve
117	Fn-422	En	Co	C	+ve	+ve	Cocci	Single & pair	-ve
118	Fn-433	En	Ri	C	+ve	-ve	Cocci	Single and pair	-ve
119	Fn-444	En	Ri	C	+ve	-ve	Cocobacili	Single	-ve
120	Fn-455	En	Co	C	+ve	+ve	Cocci	Single and Pair	-ve
121	Fn-511	En	Ri	C	+ve	-ve	Cocci	Pair	+ve
122	Fn-522	En	Ri	C	+ve	-ve	Cocci	Pair and chain	-ve
123	Fn-533	En	Ri	C	+ve	-ve	Cocci	Pair and Chain	-ve
124	Fn-544	En	Ri	C	+ve	-ve	Cocci	Pair	+ve
125	Fn-555	En	Ri	C	+ve	-ve	Cocci	Short chain	-ve
126	Fn-611	En	Ri	C	+ve	-ve	Cocci	Pair and chain	-ve
127	Fn-622	En	Ri	C	+ve	-ve	Cocci	Pair and chain	-ve

128	Fn-633	En	Ri	C	+ve	-ve	Cocobacil	Pair	-ve
129	Fn-644	En	Ri	C	+ve	-ve	Cocobacil	Pair and chain	-ve
130	Fn-655	En	Ri	C	+ve	+ve	Cocobacil	Pair and chain	-ve
131	Fn-711	En	Co	C	-ve	+ve	Cocci	Short chain	+ve
132	Fn-722	En	Ri	C	+ve	+ve	Rod	Short rod	+ve
133	Fn-733	En	Ri	C	+ve	+ve	Oval	Single	-ve
134	Fn-744	En	F	R	-ve	+ve	Cocci	Short chain	+ve
135	Fn-755	En	Ri	I	+ve	+ve	Cocci	Short chain	-ve
136	Fn-811	En	Ri	C	+ve	-ve	Cocci	Pair	-ve
137	Fn-822	En	Ri	C	+ve	-ve	Cocci	Pair	-ve
138	Fn-833	En	Ri	C	+ve	-ve	Cocci	Short chain	-ve
139	Fn-844	En	Ri	C	+ve	-ve	Cocci	Short chain	-ve
140	Fn-855	En	Ri	C	+ve	-ve	Cocci	Pair	-ve
141	Fn-911	En	Ri	C	+ve	-ve	Cocci	Pair	+ve
142	Fn-922	En	Ri	C	+ve	-ve	Cocci	Pair	+ve
143	Fn-933	En	Ri	C	+ve	-ve	Cocci	Pair	-ve
144	Fn-944	En	Co	C	+ve	+ve	Cocci	Single	+ve
145	Fn-955	En	Co	C	+ve	+ve	Cocci	Single	-ve
146	Fn-1011	En	Ri	C	+ve	-ve	Cocci	Pair	-ve
147	Fn-1022	En	Ri	C	+ve	+ve	Cocci	Pair and Chain	-ve
148	Fn-1033	En	Ri	C	+ve	+ve	Cocci	Pair and Short chain	-ve
149	Fn-1044	En	Ri	C	+ve	-ve	Cocci	Short chain	-ve
150	Fn-1055	En	Ri	C	+ve	-ve	Cocci	Pair	+ve

Forms:-Circular (C) Irregular (I) Rhizoid (R)

Elevation:-Flat (F) Raised (Ri) Convex (Co)

Margin:-Entire (En) Undulated (Un) Serrate (Se) Filamentous (Fi) ,Lobate (Lo)

Areas: - Lumme (Mj), Fentale (Fn), Adami tulu (Ad)

+ve: - Positive

-ve: -Negative