

ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

**ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA FROM 'QIBE', A
TRADITIONALLY PREPARED ETHIOPIAN BUTTER**

A Thesis Presented to the College of Veterinary Medicine and Agriculture of Addis Ababa
University in partial fulfillment of the Degree of Master of Science in Veterinary Public Health
Department of Microbiology and Veterinary Public Health

BY

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DEBRE ZEIT, ETHIOPIA

JUNE, 2012

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ACKNOWLEDGMENTS

I bow my head in deep gratitude to God, The Almighty, who is the intact and original source of knowledge and wisdom to me and all mankind. As with all the things that have happened to me, I thank God above all others for continuously blessing my life and giving me the strength to persevere during trying times.

Only next God, my deepest love and respect goes to my parents (Dad and Mom) for their never ending care and support ever since I remember. May God give them long life and good health to see more of their fruit blossom.

I like to thank my advisor Dr. Tesfaye Sisay for his unreserved comment on the contents of the paper since the time of the proposal writing and for his kind help and supervision of my activities and for laying down the necessary laboratory facilities suited for my research.

I would like to extend my thank to my co-advisor Dr. Nesredin Hussein who advised me to work on this interesting subject matter and who guided me through the development of this thesis work as well as for providing me with the Lab materials essential for the research without of which this would not have been possible.

I would also like to pass my heartfelt appreciation to Ato Muluken Tekle, W/rt Tesfanesh Moges and W/rt Zenebech for their indefatigable technical assistance with in the Microbiology Laboratory.

I am also grateful to my colleague Dr. Tenagne Misganaw for his help in finding some of the microbiological growth media in the market.

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ABBREVIATIONS

CFU	Colony Forming Unit
EPS	Exopolysaccharide
FAO	Food and Agriculture Organization
FOSHU	Foods for Specified Health Use
GRAS	Generally Accepted As Safe
HACCP	Hazard Analysis Critical Control Point
ILCA	International Livestock Center for Africa
ILCAIA	International Livestock Center for Africa Internal Agitator
LAB	Lactic Acid Bacteria
LD ₅₀	Lethal Dose 50
MOA	Ministry of Agriculture
MRS	de Mann Rogosa and Sharpe medium
NSLAB	Non Starter Lactic Acid Bacteria
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
Rep-PCR	Repetitive Sequence based Polymerase Chain Reaction

ABSTRACT

'Qibe' is a traditionally prepared Ethiopian butter made by churning fully soured whole cow's milk. In this study, 'Qibe' samples originating from different parts of the country (Gojjam, Sidamo, Jimma, Sheno and Wolega) and sold at Merkato butter market have been used. The study focused on the isolation and identification of Lactic acid bacteria (LAB) from 'Qibe'. For this purpose, six different butter retail shops were chosen. LAB were isolated based on their Gram stain and catalase test reactions. They were identified to the genus level based on their growth difference at different temperature levels (10°C, 21°C, and 45°C), NaCl concentrations (4%, 6.5%), tolerance to 60°C for thirty minutes and their reaction to citrate utilization as their sole energy source. Accordingly, 56 typical isolates of lactic acid bacteria (LAB) were selected out of the 87 isolates from samples of cow's butter ('Qibe'). They were further classified into 5 different genera: *Pediococcus*, *Streptococcus*, *Lactobacillus*, *Enterococcus* and *Lactococcus* (44.6%, 21.4%, 21.4%, 12.5% and 5.3%, respectively). The highest proportion of *Pediococci* (48%), *Streptococci* (41.6%) and *Lactococci* (50%) were identified from Sidamo, Wolega and Wolega isolates, respectively. An increment in the diversity of the LAB species was also noticed in aged/ripe (three different species of LAB in 1.5 month and 2 months old Gojjam and Wolega samples, respectively, as compared to only one species of LAB identified from a 2 weeks old Sheno and Sidamo samples) butter than in fresh 'Qibe' samples. The results showed that *Pediococcus*, *Streptococcus* and *Lactobacillus* were the predominant genera in this traditionally produced butter. However, identification of these isolates to the species level and assessing the technological performance of these predominant isolates is essential and needs further investigation.

Key words: *Butter*, *Enterococcus*, *LAB*, *Lactobacillus*, *Lactococcus*, *NSLAB*, *Pediococcus*, *Streptococcus*, 'Qibe',

1. INTRODUCTION

Interest in microorganisms as a component of biological diversity has been renewed in recent years. The interest in the microorganisms occurring in foods is primarily due to the biotechnological potential of new bacterial species and strains (Idoui and Karam, 2008). Lactic acid bacteria (LAB) are among the best studied microorganisms. Detailed knowledge of a number of physiological traits has opened new potential applications for these microorganisms in the food industry, while other traits might be beneficial for human health. Important new developments have been made in the research of LAB in the areas of multidrug resistance, bacteriocins and quorum sensing, osmoregulation, proteolysis, autolysins and bacteriophages. Recently, progress has been made in the construction of food grade genetically modified LAB (Konings *et al.*, 2000). LAB were first isolated from milk and have since been found in such foods and fermented products as meat, milk products, vegetables, beverages and bakery products (Liu, 2003).

A wide variety of products are manufactured by processing raw milk into dairy products. Among them, butter is one of the primarily fat sources and an important source of dietary energy. It has been produced since ancient times and was an internationally traded commodity as early as the 14th century (Rady and Badr, 2003). The microbiological properties of butter made from different milks have been extensively studied by many researchers (Idoui *et al.*, 2009). Some researchers reported that lactic acid bacteria (Lactococci, Lactobacilli, Enterococci, Streptococci and Leuconostocs) were isolated from natural butters (Sagdic *et al.*, 2002). Idoui and Karam (2008) have reported that *Lactobacillus plantarum* was the predominant species in traditionally prepared butter from cow milk.

In Ethiopia, some research has been performed on the antimicrobial activity of LAB isolated from traditional Ethiopian fermented foods and beverages. Idris (1999) reported the antimicrobial activity of LAB isolated from 'Awaze' and 'Datta'. The antimicrobial activity of LAB isolated from two traditionally fermented beverages ('Borde' and 'Shameta') was also examined by Girum *et al.* (2005). The microbiological safety of two widely consumed LAB fermented

Ethiopian foods, 'Kocho' and 'Tef', against some pathogenic and spoilage bacteria have been found by Ayele (1998). Mekonen and Mogessie (2005) reported that the decrease in the population of *E. coli* O157:H7 in fermenting 'Ergo' could have been due to the antagonistic activities of the LAB through their metabolites and resulting low pH. 'Ergo' as a promising source of antimicrobial substance-producing LAB have been reported by Assefa *et al.* (2008). The major lactic acid bacteria isolated from traditionally fermented milk 'Ititu' in the southern part of Ethiopia was identified to be *Lactobacillus plantarum* (Ashenafi, 1994). Almaz *et al.* (1999) reported that 'Ergo' fermentation is carried out by lactic acid bacteria belonging to the genera *Lactococcus*, *Streptococcus*, *Leuconostoc* and *Lactobacillus*. They also observed that *Micrococcus spp.*, coliforms and spore formers were also present in fairly high numbers during the first 12-14 hrs. of fermentation.

The major traditional butter produced in Ethiopia is cow's milk butter, called 'Qibe'. Rural people still produce 'Qibe' by traditional methods using primitive utensils. The raw material is cow's milk, after spontaneous coagulation it is churned and then the "Qibe" is recovered. To our knowledge, there is still no information about the lactic acid bacteria population of traditionally prepared butter 'Qibe' from cow's milk in Ethiopia.

OBJECTIVES

The primary objective of this study was to isolate and identify lactic acid bacteria (LAB) from the traditionally produced Ethiopian cow butter, called 'Qibe'.

The following were the specific objectives of the research work

- Isolation of lactic acid bacteria from butter, 'Qibe'.
- Identification of the genera of lactic acid bacteria present in 'Qibe'.
- Determining the type of lactic acid bacteria present in the various types of butter drawn from different origins

2. LITERATURE REVIEW

2.1. Milk production in Ethiopia

The major species used for milk production in Ethiopia are cattle, camel and goats. Total milk production in Ethiopia is estimated to be 1.19 million metric tons. Cattle produce 83% of the total milk and 97% of the cow milk comes from indigenous cattle breeds (MOA, 1997). Although milk production is increasing by 1.2% per annum, the demand-supply variance for fresh milk is ever widening and the per capita consumption diminishing. The dairy industry in the country is constrained by several technical and economic factors (Fekadu, 1994) and the national milk production remains among the lowest in the world, even by African standards (Zegeye, 2003). Ethiopia is not self-sufficient in milk and a considerable amount of foreign exchange has to be spent on the import of dairy products. Per capita consumption of milk is low in Ethiopia (17kg) and Tanzania (22kg) compared with Kenya (80kg) and the average for Africa (26kg) (Gebre Egziabher *et al.*, 2000).

Livestock are raised in all of the farming systems of Ethiopia by pastoralists, agro-pastoralists and crop-livestock farmers. According to Tsehay (2002), milk production systems can be broadly categorized in to urban, peri-urban and rural milk production systems, based on their location (Table 1). Milk is produced in all the agricultural production systems in Ethiopia. The bulk (98%) of the Milk is produced in rural areas by subsistent farmers (Tsehay, 1998). A survey conducted by MOA (1997) in highland peri-urban areas indicated that in most peri urban areas 74.6% of the milk produced is either used at home or marketed in liquid form while 17.5% fed to calves, 8% churned and marketed in to butter oil state and 0.3% is wasted. Specifically, in Arsi 38.5% and in Gojjam 19% of the milk is converted into butter and marketed. In Addis Ababa, 94.5% of the milk produced in intra-urban and peri-urban areas is marketed in fresh milk form. Tsehay (2002) described that urban milk production system inside and around Addis Ababa consists of 5,167 small, medium and large dairy farms producing about 35 million liters of milk annually.

Table 1. Structure of demand for milk products in Ethiopia, 2000.

Milk products	Households			
	Rural (%)	Peri-urban (%)	Urban (%)	Total (%)
Raw milk consumed by calves	32	13	9	32
Raw milk consumed by humans				
Farm households	15	8	10	15
Marketed	2	59	61	4
Butter	41			40
Cheese	9	20	8	9
Pasteurized milk	1	0	12	1
Total milk equivalent (%)	100	100	100	100
Litres ($\times 10^6$)	1115	15	20	1135

Source: Tsehay (2002)

Out of the total volume of milk produced in and around Addis Ababa, 73%, 10%, 9.4%, 7.6% were marketed, left for household consumption, goes to calves and processed in to butter and 'ayib' (Ethiopian cottage cheese), respectively (Azage and Alemu, 1998). In general, according to some investigation by the years 1985 and 1998 the total milk available, share of cow's milk and the share of imported milk from the total were summarized in Table 2.

Table 2. Total milk production and imports for Ethiopia, 1985 and 1998.

Milk availability and percentage	Years	
	1985	1998
Total milk availability ($\times 10^3$ tones)	1125	1170
Percentage of total milk availability		
Cows' milk	60.7%	80.1%
Milk of other species	19.6%	19%
Net imports	19.7%	0.9%

Source: Nicholson et al., 2001

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 Cunningham, 1991).

2.2. Milk products in Ethiopia

Milk has an essential role in most diets world-wide. Consumption of milk and fermented milk products is widespread and yet increasing. In developed countries where there is an established dairy industry, new products are being developed to sustain consumer interest (Yaman and Elmali, 2010). The rural communities in Ethiopia produce fermented milk by traditional methods. The major fermented milk products produced by small-holder farmers include 'Qibe' (traditional butter), 'Ergo' (fermented sour milk), 'Ititu' (Fermented milk curd), 'Neter kibe' (spiced butter), 'Ayib' (cottage cheese), 'Arerra' (Sour defatted milk) and 'Aguat' (whey) (Assefa *et al.*, 2008).

2.3. Traditional butter ('Qibe') preparation in Ethiopia

Milk fat is one of the major products in the dairy industry and has been primarily used in the production of butter. It is a complex fatty acid composition that results in a unique mixture of triglycerides with a wide range of molecular weights and degrees of saturation. Besides fats, butter contains small percentages of protein, milk sugar and water which make it a suitable substrate for microorganisms. Butter is widely used as a seasoning in cooking, suggesting that it plays an important role in the formation of flavors during heating (Idoui *et al.*, 2009).

Butter making in Ethiopia is closely associated with fermented milk as most of the traditional butter is made by churning fully soured whole milk. In Ethiopia traditional butter is not made from cream. The use of natural fermentation is the common means of achieving the necessary souring either in the formation of a fermented milk product ready for consumption or in an acidification or souring in a product preparation. It is clear that many variations in the characteristics, quality and acceptability of traditional milk products are inevitable due to the unregulated nature of natural fermentation (FAO, 1990).

The techniques of churning differ from place to place depending on the size and make of the churn. For example, O'Mahony and Ephraim (1985) identified four different methods of butter making in Ethiopian highlands: 1) The churn is placed on the floor, on a soft pad of material such as sheep skin or straw, tilted at an angle of 75° to the horizontal, and rocked back and forth; 2) The churn is hung on a tripod or door post and swung to and fro; 3) The churn is rocked on the lap, and 4) The churn is shaken with both hands. Zelalem (1999) also identified three different butter making methods in the central highlands of Ethiopia: 1) Sour milk is agitated by placing the churn (clay pot) on the floor and rolling it back and forth; 2) Sour milk is stirred with *mesbekia* (stick with three to six finger like projections at one end) by inserting it in the sour milk inside the clay pot and using the palms of both hands to rotate the stick. In this case the clay pot is not moved; 3) The sour milk is initially stirred for some time with a *mesbekia* and then agitated by rolling the sour milk in the clay pot back and forth until fat is recovered in the form of butter. Accordingly their efficiency of churning may also differ. But there is no documented report on different methods of butter making in the Mid Rift Valley of Oromia.

Traditional churning is time-consuming and labor intensive, and results in considerable losses of fat in the buttermilk (O'Mahony and Ephraim, 1985). In order to alleviate these problems, the International Livestock Center for Africa (ILCA) tried to modify the traditional churning clay pot to the internal agitator called International Livestock Center for Africa Internal Agitator (ILCAIA). ILCAIA consists of a central shaft, paddle blades, and restraining shafts, which fix the agitator in position. The paddle blades are fitted on the central shaft in situ. Pulling on a rope wound around the main shaft of the agitator, churning is effected and it has an average churning time of 61 minutes and 0.4 percent fat content of buttermilk (O'Mahony and Peters, 1987).

2.4. Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) are characterized as Gram - positive, cocci, cocco-bacilli or rods, usually non-motile, non-sporulating bacteria that produce lactic acid as a major or sole product of fermentative metabolism (Nair and Surendran, 2005). In most cases they are anaerobic, microaerophilic or aerotolerant in their oxygen demands. Although some may produce pseudo catalase when grown at low sugar concentrations, they are generally catalase and oxidase negative. They are chemo-organotrophic and grow in complex media. Lactic acid bacteria (LAB), which are primarily used by the dairy industry, are extensively utilized in the fermentation of wide variety of food products and are known for their preservative and therapeutic effects (Karna *et al.*, 2007). In addition, they strongly determine the flavor, texture and frequently, the nutritional value of food and feed products. However the application of well-studied starter cultures has been established for decades (Tserovska *et al.*, 2002).

2.4.1. Classification

LAB are united by a constellation of morphological, metabolic and physiological characteristics. Lactic acid bacteria (LAB) consist of a number of bacterial genera within the phylum *Firmicutes*. The genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* are recognized as LAB (Ercolini *et al.*, 2001).

Members of LAB share the property of being Gram-positive bacteria that ferment carbohydrates into energy and lactic acid. Depending on the organism, metabolic pathways differ when glucose is the main carbon source as: homofermentative and heterofermentative bacteria (Jay, 2000).

The taxonomy of LAB (Fig. 1) based on comparative 16S ribosomal RNA (rRNA) sequencing analysis has revealed that some taxa generated on the basis of phenotypic features do not correspond with the phylogenetic relations (Zoetendal *et al.*, 2002). LAB from food and their current taxonomical status have been described by Gonzalez *et al.* (2000). Molecular techniques, especially polymerase chain reaction (PCR) based methods, such as rep-PCR fingerprinting and

restriction fragment length polymorphism (RFLP) have been used in the taxonomy of LAB (Holzapfel *et al.*, 2001).

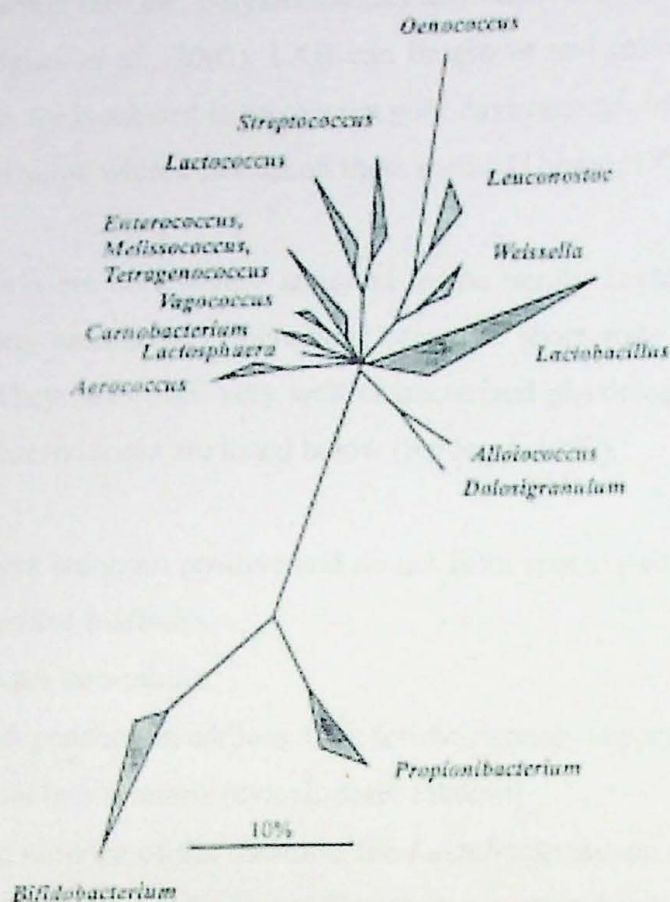


Figure 1. Consensus tree based on comparative analysis of the 16S rRNA gene of Lactic acid bacteria.

Source: Holzapfel *et al.*, 2001

2.4.2. Morphological and physiological characteristics of LAB

Members of LAB share the property of being Gram-positive, cocci, rods and cocco-bacilli bacteria that ferment carbohydrates into energy and lactic acid (Jay, 2000). Depending on the organism, metabolic pathways differ when glucose is the main carbon source: homofermentative

bacteria such as *Lactococcus* and *Streptococcus* yield two lactates from one glucose molecule, whereas the heterofermentative, i.e. *Leuconostoc* and *Weissella*, transform a glucose molecule into lactate, ethanol and carbon dioxide. In addition, LAB produce small organic compounds that give the aroma and flavor to the fermented product. LAB also produce acetaldehyde, hydrogen peroxide, diacetyl, carbon dioxide, polysaccharides and bacteriocins, some of which may act as antimicrobials (Rodríguez *et al.*, 2003). LAB can be grown and enumerated on agar plates, as long as the agar plates are incubated in an oxygen poor environment. Most LAB grow on MRS or Rogosa agar and form snow white colonies on these media (Theron, 1999).

The lactic acid bacteria are collectively assigned to the family *Lactobacteriaceae*. This group exhibits heterogeneous morphology including long and short rods as well as cocci of the *Streptococcus* type. They have been very well characterized physiologically (Table 3). Some of the features of *Lactobacteriaceae* are listed below (Schlegel, 1997).

- All its members are gram positive and do not form spores (with the solitary exception of *Sporolactobacillus inulinus*)
- Most of them are non-motile
- They all are dependent on carbohydrate for their energy supply and excrete lactic acid
- They do not contain haemins (cytochrome, catalase)
- In spite of the absence of the haemins, the *Lactobacteriaceae* especially the Streptococci can grow in the presence of oxygen. Thus they are anaerobes but aerotolerant
- They require complex media containing several growth factors such as vitamins, lactoflavin, thiamine, nicotininc acid, folic acid, biotin, pantothenic acid etc.

Thus, lactic acid bacteria may be regarded as metabolic cripples that have lost their ability to synthesize a number of metabolites as a consequence of their specialization for growth on milk. On the other hand they have the ability that most microorganisms lack; they can utilize lactose. Lactose apparently does not occur in the plant kingdom and it is produced, excreted and ingested in milk by mammals. The utilization of lactose by microorganisms can thus be regarded as an adaptation to ecological conditions in the mammalian digestive tract. Lactose is a disaccharide that must be hydrolyzed before it can enter the catabolic pathway for hexoses (Schlegel, 1997).

Table 3. Differential characteristics of lactic acid bacteria based on morphology and physiology

Genus	<i>Lactobacillus</i>	<i>Enterococcus</i>	<i>Lactococcus</i>	<i>Leuconostoc</i>	<i>Pediococcus</i>	<i>Streptococcus</i>
Characteristic						
Morphology	rods	cocci	cocci	cocci	cocci in tetrads	cocci
CO ₂ from glucose*	±	—	—	+	—	—
Catalase test	—	—	—	—	—	—
Growth						
at 10°C	±	+	+	+	±	—
at 45°C	±	+	—	—	±	±
in 6.5% NaCl	±	+	—	±	±	—

+ Positive; - negative; ± varies between species

*test for homo- or heterofermentation of glucose: - homofermentation, + heterofermentation

Source: Todor (2011)

Kluyver divided LAB into two groups based on the end product of glucose metabolism (Fig. 2); those that produce lactic acid as the only product of glucose fermentation are designated as homofermentative. The homofermentative pattern is observed when glucose is metabolized but not necessarily when pentose sugars are metabolized, for some, homolactics produce acetic and lactic acids when utilizing pentose. Also the homofermentative character of homolactics may be shifted for some strains by altering cultural conditions such as glucose concentration, pH and nutrient limitation (Schillinger and Luke, 1987).

The homolactics are able to extract twice as much energy from a given quantity of glucose as are the heterolactics. Those lactics that produce equal molar amounts of lactate, carbon dioxide and ethanol from hexoses are designated as heterolactics. All members of the genera *Pediococcus*, *Streptococcus*, *Lactococcus* and *Vagococcus* are homofermenters, along with some of the *Lactobacilli*, while all *Leuconostoc* spp, as well as some *Lactobacilli* are heterofermenters. The heterolactics are more important than the homolactics in producing flavor and aroma components such as acetylaldehyde and diacetyl (Schillinger and Luke, 1989). Although LAB are comprised

of 11 genera, only six of them are dairy associated. These are *Lactococcus*, *Enterococcus*, *Streptococcus*, *Leuconostoc*, *Pediococcus* and *Lactobacillus* (Garvie, 1984).

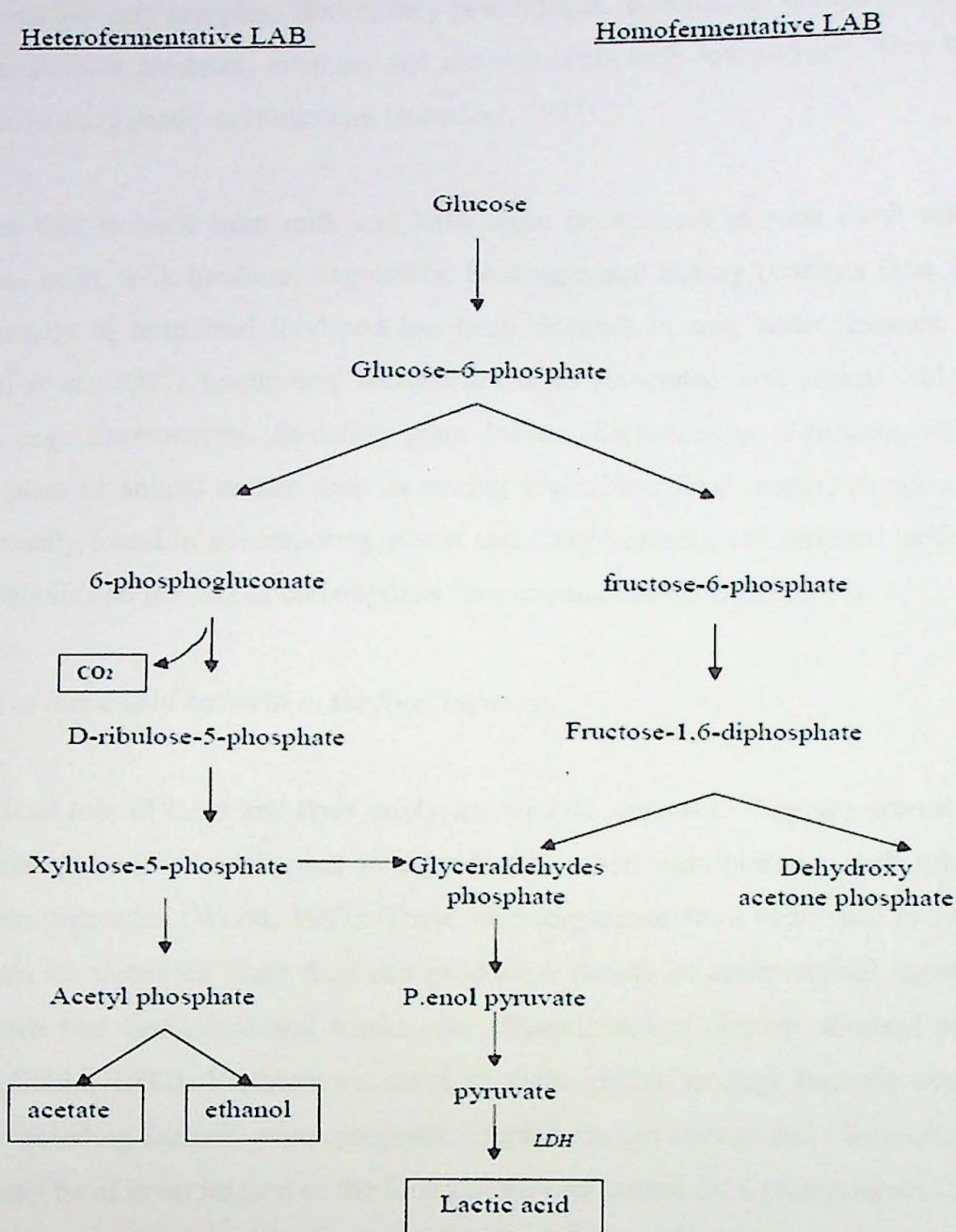


Figure 2. Glucose utilization metabolic pathways of LAB

Source: Garvie (1984)

2.4.3. Occurrence

The distribution of lactic acid bacteria in nature is related to their high demand of nutrients and their type of energy generation (Schlegel, 1997). LAB are widespread in nature, their nutritional requirements are very complex. Hence, they predominate habitats that are rich in carbohydrates, protein breakdown products, vitamins and environments with low oxygen. This confirms the prevalence in dairy products (Stiles and Holzapfel, 1997).

LAB were first isolated from milk and have since been found in such foods and fermented products as meat, milk products, vegetables, beverages and bakery products (Liu, 2003). LAB occur naturally in fermented food and has been detected in soil, water, manure and sewage (Holzapfel *et al.*, 2001). Lactic acid bacteria are often associated with animal oral cavities and intestines (e.g. *Enterococcus faecalis*); plant leaves (*Lactobacillus*, *Leuconostoc*) as well as decaying plant or animal matter such as rotting vegetables, fecal matter, compost, etc. These bacteria usually found in decomposing plants and dairy products and produce lactic acid as the major metabolic end product of carbohydrate fermentation (Sabia *et al.*, 2004).

2.4.4. Use of lactic acid bacteria in the food industry

The beneficial role of LAB and their safety are well documented. They are extensively used in food processing, such as in dairy and meat products for their contribution to shelf life, texture and organoleptic properties (Wood, 1997). These microorganisms have been used in food and feed preservation for centuries since they can produce a variety of antimicrobial agents, including organic acids like lactic acid and acetic acid, ethanol, carbon dioxide, diacetyl and hydrogen peroxide (Gould, 1992). Furthermore, some of them inhibit spoilage bacteria and food borne pathogens including *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium botulinum* and thus may be of great interest to the food industry as natural food preservatives (Stiles, 1996). LAB are important commercially in the processing of meat, alcoholic beverages and beverages. The products include sausages, cured hams, wines, beer, fortified spirits, pickles and sauerkraut (Jay, 1992).

To date, exopolysaccharides (EPSs) produced by Lactic acid bacteria (LAB) have received increasing interest because of their GRAS (generally regarded as safe) status, and their rheological (LAB) properties in food to improve the texture of fermented products (Adebayo-tayo and Onilude, 2008). LAB are associated with humans, animals, dairy products, fermented beverages and plant material. Generally, dairy product materials constitute a major source for isolation and screening of lactic acid bacteria (Kacem *et al.*, 2003).

2.4.5. Role in food preservation

Lactic acid bacteria (LAB) are widely utilized to produce fermented foods contributing 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). 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 borne 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 as they enhance the shelf life of the food (Soormro *et al.*, 2002). LAB have been used as a flavoring and texturizing agent as well as a preservative in food for centuries and are now added as starters in food. LAB, such as Lactobacilli, *L. lactis*, and *Streptococcus thermophilus*, inhibit food spoilage and pathogenic bacteria and preserve the nutritive qualities of raw food material for an extended shelf life (O'Sullivan *et al.*, 2002).

The preservative effect of LAB during the manufacture and subsequent storage of fermented foods is mainly to the acidic conditions that they create, converting carbohydrates to acetic and lactic acids in the food during their development (Hiromi *et al.*, 1997).

2.4.6. Lactic acid bacteria in milk fermentation

Lactic acid bacteria frequently termed as “Lactis” are basic starter cultures with widespread use in the dairy industry for cheese making, cultured buttermilk, cottage cheese and cultured sour

cream (Jay, 1986). Identifying and providing a practical means of using appropriate starter cultures is advantageous due to the competitive role of microorganisms and their metabolites in preventing growth and metabolism of unwanted microorganisms. A strong starter may reduce fermentation times, minimize dry matter losses, avoid contamination with pathogenic and toxigenic bacteria and molds and minimize the risk of incidental microflora causing off flavor, etc. and technology (Holzapfel, 2001).

During biblical times, people in Middle East discovered yoghurt. They found that when milk was left in a warm place, it thickened and developed a different, tart flavor. More importantly, it kept better than fresh milk. It was centuries later that scientists discovered that tiny bacteria made yoghurt. Most yoghurt is made from either *Lactobacillus bulgaricus* or *Streptococcus thermophilus*. Once the bacteria are added to milk, these bacteria consume the milk sugars and undergo fermentation, much like the yeast in bread. The benefit of having a fermented milk product is that so much acid is produced by these microorganisms that few other potentially harmful microorganisms can grow in this acid yoghurt environment. 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 on the basis of their growth optimum. Mesophilic lactic acid bacteria have an optimum growth temperature between 20°C and 30°C and the thermophilic have their optimum between 30°C and 45°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 which originated from western and northern European countries (Wauters *et al.*, 2002). The end product in the fermentation pathway is the production of more than 90% lactic acid, which is responsible for the sharp refreshing test, preservation of milk products and gel formation. Although lactic acid is the predominant metabolite of glycolysis, other metabolites which may be present in small concentrations are also important for product's flavor (Urbach, 1995).

2.4.7. Lactic acid bacteria as antimicrobials and probiotics

Lactic acid bacteria are the most used microbial groups in fermentation industries. Lactic acid bacteria are involved in several aspects of human life, from food processing to health care. The antibacterial properties of lactic acid produced by these bacteria are exploited *in vitro* for food preservation and *in vivo* through probiotic formulations (Bayane *et al.*, 2010).

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 (Cardruci and Citak, 2005).

Recognition of the importance of the intestinal microbiota to health has led to increasing interest in manipulating the composition and activity of the microbiota to improve both human and animal health. Examples of bacteria demonstrated to have beneficial effects include *Lactobacillus*, *Bifidobacterium*, *Escherichia coli* Nissle 1917, *Clostridium butyricum*, *Streptococcus salivarius thermophilus* and a nonpathogenic yeast *Saccharomyces boulardii* (Sartor, 2004).

Probiotics are defined as living microorganisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition (Tannock, 2002). Mechanisms by which probiotics exert their therapeutic effects include modulation of barrier function, mucosal trophic action, inhibition of pathogenic bacteria, blockade of epithelial attachment and invasion by pathogenic bacteria, modulation of intestinal cytokine production, anti-inflammatory properties, and enhancement of digestion and absorption of food (Pishva *et al.*, 2009). Some physiological characteristics are of interest for their function as probiotics, a precondition of which is survival in the gastrointestinal tract and low pH, bile and their temperature growth (Fuller, 1989).

The use of LAB as a probiotic requires a safety assessment. The functional properties of the strains should be well studied and documented. Generally recognized health-promoting properties are non-pathogenic behavior, the ability to persist with in the GI tract and adhesion, and the

ability to modulate immune responses (Holzapfel et al., 2001). Gibson and Fuller (2000) pointed out the importance of considering the possible side effects of probiotics on the consumer, e.g. bloating or blocking the normal functional gut transit. *Lactobacilli* and *Lactococci* commonly hold a GRAS status. Japan legally recognizes functional foods (Foods for Specified Health Use, FOSHU) (Sanders, 2003). Lethal dose (LD₅₀) of LAB was measured for mice by oral administration and found to be $>10^{11}$ cfu/kg, depending on the strain (Ishibashi and Yamazaki, 2001). The safety of two *Bifidobacterium longum* strains of human origin was evaluated on healthy adult volunteers: no side effects were reported and the immune parameters measured remained without undesirable changes (Makelainen et al., 2001).

3. MATERIALS AND METHODS

3.1. Description of the study area

The study was conducted in Addis Ababa city, at Merkato market place. Samples of butter were drawn from wholesale and retail shops at Merkato butter market. Addis Ababa is the capital city of Ethiopia (latitude and longitude; 8°59'N 38°48'E) with an altitude of 2,324 m above sea level. The average annual rainfall in Addis Ababa is 1,178 mm. The main wet season lasts from June to September, causing about 70% of the annual rainfall with the highest peak in August. Another small peak of rainfall is observed in March and April. The minimum and maximum temperature is 8°C and 23°C, respectively.

3.2. Survey

A cross-sectional preliminary survey was conducted in the study area to assess the transport, distribution, storage, package and sell of the product. Besides, the origin of the product was assessed. In order to address the study objectives, six retail shops were randomly selected from Merkato butter market.

3.3. Experimental work

3.3.1. Sample collection

The material was cow butter, traditionally produced and named “Qibe”. Throughout the sample collection time a total of 22 different butter samples weighing about 2.1 kg were collected from randomly selected six retail shops from Merkato butter market. The samples were then transported in an ice box to the Microbiology laboratory of SVM, AAU in sterile plastic bags and

stored at +4°C until used for further analysis. While the samples were being collected the sellers were asked about the origin and age of the butter (Table 4).

Table 4. The source, age and number of samples collected from the Merkato butter market.

Source	Market	Age	Number of samples/source
Gojjam – tilili	Merkato	2 weeks	4
Gojjam – tilili	Merkato	1.5 month	3
Sidamo	Merkato	2 weeks	2
Sidamo	Merkato	1 year	3
Jimma	Merkato	2 weeks	2
Jimma	Merkato	3 weeks	3
Sheno	Merkato	2 weeks	2
Wolega	Merkato	2 months	3

3.2.2. Sample preparation and enrichment

Samples of butter ‘Qibe’ from each sample were centrifuged at 3,000 rpm for 15 minutes. Then, 1ml of the intermediate liquid part was taken and 1:10 serial dilutions from 10^{-1} up to 10^{-7} were prepared as described by Idoui *et al.* (2009).

3.2.3. Isolation of Lactic acid bacteria

One hundred µl of sample from the 10^{-4} to 10^{-7} serial dilutions were cultured in duplicate on each of de Man, Rogosa and Sharpe (MRS) and M17 agar plates by streak plate technique and incubated at 37°C in a 6% CO₂ gas injecting incubator for 72 hours.

The preparation of MRS and M17 agar media and MRS broth media was done according to the manufacturers’ manual (Annex V). MRS agar and Medium are designed for the cultivation, enrichment and isolation of *Lactobacillus* species from all types of materials (De Man *et al.*,

1960). M17 Agar is used for isolating and enumerating lactic Streptococci in yoghurt, cheese starters and other dairy products (Richter and Vedamuthu, 2001). MRS and M17 broth media consist of the same ingredients with their respective agar media. The only difference is their respective agar media have agar, a solidifying agent.

The desired glistening colonies (87) were randomly picked up from the MRS and M17 agar (i.e. from each) plates by sterile loops. The colonies were purified by successive streaking on the appropriate agar media (MRS and M17) before being subjected to characterization. Purification of the culture colonies was confirmed after successive sub-culturing and examining for their Gram reaction, cell and colony morphology and catalase reaction. Those that are characterized as lactic acid bacteria were used for further identification.

Gram staining

The gram reaction of the isolates was determined by light microscopy after Gram staining. LAB are known to be Gram positive. It means that they give the color of the first stain, violet, by gram staining. Cultures were grown in appropriate conditions at 37°C for 24 h under anaerobic conditions. Then, cells from thus cultures were picked up by sterile wire loops and put on a clean microscopic slide. Gram staining procedure was applied. Then under microscopy violet colored isolates, Gram positives, were identified and determined.

Catalase test

Catalase test was performed to isolates in order to see their catalase reactions. For this purpose, a drop of 3% solution of hydrogen peroxide (H_2O_2) was placed on a clean microscope slide, and a 24 hour old pure culture colony from the MRS and M17 agar plates was picked up using sterile wire loop and mixed with the drop of hydrogen peroxide. The evolution of gas bubbles from the mix was indicative of positive test, and the absence of it implied a negative reaction. The isolates which did not give gas bubbles were chosen, since LAB are catalase negative.

3.3.4. Biochemical characterization and identification of the isolates

After obtaining pure colony, colonies were randomly selected and streak plating was done which were subsequently stored in two different conditions including at 4°C on MRS and M17 agar plates and at -20°C in MRS broth supplemented by 10% glycerol for further use (Mathara *et al.*, 2004). All strains were initially tested for Gram reaction, catalase test and cell and colony morphology on MRS and M17 agar plates.

i. Growth at different temperatures

The growth of the isolates at some selected temperatures was determined. For this purpose, purified fresh colony was inoculated in to a sterile MRS broth media and incubated for 72 hours at 10°C, 20°C, and 45°C. During this incubation time cell growth at any temperature was observed by the change in the turbidity of the medium as compared with a negative control.

ii. Growth at different NaCl concentrations

Isolates were tested for their tolerance against different NaCl concentrations. Saline solutions of 4% and 6.5% NaCl were selected to conduct this test. Fresh colonies were inoculated in to tubes containing 5ml of each of the saline solutions and incubated for 72 hours at 37°C. The change in the nature of the medium from the original clear medium to a turbid medium was indicative of bacterial growth and multiplication. The turbidity indicated the isolates taken have grown and are tolerant to that concentration of NaCl.

iii. Tolerance to 60°C

The likelihood of the bacterial isolates to stay alive for thirty minutes under 60°C was also evaluated. For this, pure colony containing MRS and M17 agar plates were incubated at 60°C for thirty minutes. After the due time, individual colonies from each plate were streak plated on another sterile media and incubated at 37°C for 72 hours. Bacterial growth, which was indicative of tolerance to an earlier incubation at 60°C, was confirmed visually by the presence of visible grown culture colonies.

iv. Citrate test

The citrate test utilizes Simmon's citrate media to determine if a bacterium can grow utilizing citrate as its sole carbon and energy source. Simmon's media contains bromthymol blue, a pH indicator with a range of 6.0 to 7.6. Un inoculated Simmon's citrate agar has an intermediate green color. Growth of bacteria in the media leads to development of blue color (positive citrate). Fresh culture colonies were inoculated in to a Simmon's citrate agar media containing test tubes and incubated for 48 h at 37°C. Development of blue color was taken as positive for citrate test.

3.4. Data analysis

The data collected during the laboratory experiments were analyzed using the descriptive statistics and percentage comparison procedure of the statistical package for social science (SPSS V. 12.0).

4. RESULTS

4.1. Survey results

From the preliminary survey conducted in the study area, poor and unhygienic handling, storage, transport and sell of the product has been observed. In most of the cases, no washing of hands and the materials used for storage, transport of butter was seen. This may be due to lack of awareness on the perishability of the product as well as carelessness. Among the commonly observed bad handling practices are that of the seller/merchant inserting his/her fingers in to the product every now and then for weighing the product, absence of hand washing, storing butter side by side with other products like onions, garlic, and the like, leaving the container open, mixing long ripened butter with fresh ones.

4.2. Isolation of lactic acid bacteria

A series of four sub culturing steps on MRS and M17 agars, Gram reaction, catalase test and observation for the cellular morphology resulted in approximately 87 probable LAB isolates. The number of LAB isolated from this initial isolation step from butter 'Qibe' and their morphological and biochemical properties are presented in Table 5. Lactic acid bacteria were picked up from the MRS and M17 agar growth media according to their characteristic snowy white transparent colonies (Annex II). Gram positive, catalase negative, cocci, cocco-bacilli, or rod-shaped isolates with characteristic colonial arrangements on MRS and M17 growth media were considered as lactic acid bacteria (Hernandez *et al.*, 2005).

Table 5. Initial purification result after a series of sub culturing steps, Gram and catalase test.

Source	Gram reaction		Catalase		Cell shape		
	+ve	-ve	+ve	-ve	Co	Cb	Ro/b
Gojjam	28	4	8	24	17	5	10
Sidamo	17	1	4	14	10	2	6
Jimma	11	1	3	9	8	2	2
Sheno	6	1	2	5	4	1	2
Wolega	16	2	5	13	11	1	6
Total	78	9	22	65	50	11	26

Cell shape: - Cocci (Co), Cocco-bacilli (Cb), Rod (Ro)

4.3. Physiological and biochemical characteristics of the LAB isolates

From the 87 bacteria isolated from 22 samples, 56 isolates remained at the end of the isolation, after the loss of unstable isolates during purification and sub culturing steps. Twenty of which are from Gojjam, twelve from Sidamo, eight from Jimma, twelve from Wolega, four from Sheno samples (Table 6).

Table 6. Percentage of LAB isolated from each source.

Source	Number of samples	Number of LAB isolated	% of LAB isolated
Gojjam	7	20	35.7
Sidamo	5	12	21.4
Jimma	5	8	14.3
Sheno	2	4	7.1
Wolega	3	12	21.4
Total	22	56	100%

After a series of sub-culturing process and confirming the identity of the isolated bacteria to be lactic acid bacteria by their Gram reaction, catalase test, colonial characteristic (Annex II) and their cellular morphology, various physiological and biochemical tests had been performed to identify the LAB. The result for the number of LAB isolates falling for each test performed like

Citrate, growth at different temperature levels and NaCl concentrations and tolerance to 60°C for thirty minutes for the LAB isolates from each source are presented in Table 7 and Annex IV.

Table 7. The physiological and biochemical tests versus the number of LAB isolates from each source.

Source of LAB isolates	Citrate test		Growth at different temperatures				Growth at different NaCl concentration		Tolerance to 60°C	
	+	-	10°C	21°C	37°C	45°C	4%	6.5%	MRS agar	M17 agar
Gojjam	6	14	15	17	20	20	12	16	16	16
Sidamo	0	12	12	12	12	12	12	12	12	12
Jimma	8	0	4	0	8	0	4	0	5	7
Sheno	0	4	2	4	4	0	1	4	0	0
Wolega	12	0	7	3	12	0	1	6	10	10
Total	26	30	40	36	56	22	30	38	43	45

4.4. Identification of the LAB

The overall physiological and biochemical characteristics of all the 56 isolates is listed in Annex 1. Based on the characteristic physiological and biochemical results obtained from each respective test the LAB identified comprised of *Pediococcus*, *Streptococcus*, *Lactobacillus*, *Enterococcus* and *Lactococcus* with isolation rates of 44.6%, 21.4%, 21.4%, 12.5% and 5.3%, respectively. It was able to identify four different genera of LAB from the Gojjam samples. Wolega and Jimma samples exhibited three different isolates each. Only one type of LAB was identified from Sidamo and Sheno samples (Fig. 3). The highest proportion of *Pediococci* (48%), *Streptococci* (41.6%) and *Lactococci* (50%) were identified from Sidamo and Wolega isolates for the latter two, respectively. The rate at which each LAB, including the earlier, was identified from LAB isolates from each origin is described in Table 8. Out of the 56 LAB isolates 37 were cocci, 6 were cocco-bacilli and the rest 13 were rods/bacilli (Table 8).



Figure 3. The type and number of isolates identified from each source.

Table 8. The number of strains of LAB identified from each source and their characteristics.

Source	Gram reaction	Catalase	Cell shape			LAB identified				
	+ve	-ve	Co	Cb	Ro/b	<i>Pediococcus</i>	<i>Streptococcus</i>	<i>Lactobacillus</i>	<i>Enterococcus</i>	<i>Lactococcus</i>
Gojjam	20	20	14	1	4	9 (36%)	3 (25%)	4 (33.3%)	4 (100)	0
Sidamo	12	12	12	0	0	12 (48%)	0	0	0	0
Jimma	8	8	3	3	2	0	4 (33.3%)	2 (16.6%)	0	2 (66.7%)
Sheno	4	4	4	0	0	4 (16%)	0	0	0	0
Wolega	12	12	4	2	6	0	5 (41.6%)	6 (50%)	0	1 (33.3%)
Total	56	56	37	6	13	25 (100%)	12 (100%)	12 (100%)	4 (100%)	3 (100%)

Comparing the heterogeneousness of fresh 'Qibe' samples with ripened ones it was able to notice, that ripened or aged butter samples (1.5 and 2 month) exhibit a more diverse number of LAB isolates as compared to the fresh butter samples (2weeks) (Fig. 5). Hence, the study result showed Wolega (2 month old) samples and Gojjam (1.5 month old) samples exhibited three

different genera of LAB while Sidamo and Sheno (2 weeks old, for each) samples showed only one type of genera. Therefore, in fresh butter, *Pediococcus* is the prevalent LAB; whereas aging increases the number of LAB spp in the butter.

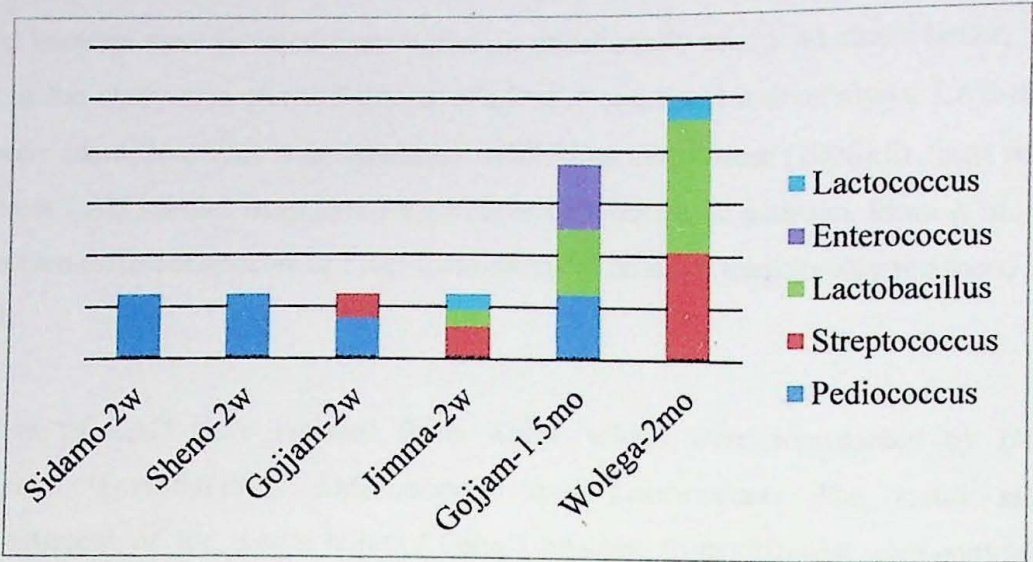


Figure 4. Diversity of LAB spp. isolated from 'Qibe' of different ages

5. DISCUSSION

In this study investigation was made on the genera of LAB occurring in traditionally made butter, 'Qibe', originating from different parts of the country and also at different ages. From our results, lactic acid bacteria were isolated from 'Qibe', a traditionally produced cow's butter. The results obtained in the study also showed that cow's butter contained a diversity of LAB and fifty six isolates were identified. This is in agreement with Idoui and Karam (2008) findings who reported five different LAB species from jijel's traditional cow butter. In addition, Idoui *et al.* (2009) also reported seven different species of LAB from sheep's 'dhan', a traditionally produced butter from sheep milk.

Five genera of LAB were isolated from 'Qibe' which were represented by *Pediococcus*, *Streptococcus*, *Lactobacillus*, *Enterococcus* and *Lactococcus*. The result showed the heterogeneousness of the cow's butter ('Qibe') brought from different agro-ecological zones. From the 56 isolates, *Pediococcus* (25), *Streptococcus* (12) and *Lactobacillus* (12) were the predominant genus in this product. This however, is not in accordance with various reports that indicate the predominant lactic acid bacteria in butter were *Lactococcus* (Guiraud, 1998). Also lots of catalase positive bacteria and yellowish fungal growth on or around the white LAB colonies (Annex III) were observed. This growth might be due to the utilization of the acidic environment by the fungal yeast cells produced by the LAB.

These results also are not in complete agreement with those reported by Sagdic *et al.* (2002) who isolated lactic acid bacteria from traditional Turkish yayik butter represented essentially by the genus *Leuconostoc* and *Enterococcus*, by Idoui and Karam (2008) who reported that the predominant LAB in jijel's traditional cow's butter to be *Lactobacillus*, *Lactococcus* and *Leuconostoc*. In addition, Idoui *et al.* (2009) reported that *Lactococcus* and *Leuconostoc* are the predominant LAB genera from sheep's butter. Also, the major lactic acid bacteria isolated from traditionally fermented milk 'Ititu' in the southern part of Ethiopia was identified to be *Lactobacillus plantarum* (Ashenafi, 1994). Almaz *et al.* (1999) reported that 'Ergo' fermentation is carried out by lactic acid bacteria belonging to the genera *Lactococcus*, *Streptococcus*,

Leuconostoc and *Lactobacillus*. The variation in the traditional practices like churning, storage of milk for fermentation, smoking would influence the type and number of lactic acid bacteria present in the final product.

Twenty five cocci isolates which were not able to use citrate as their sole energy source, the heat tolerance to 60°C for thirty minutes were considered to be *Pediococcus*. This genus constituted about 44.6% of the total isolates. It is predominant in the samples taken from Sidamo and Sheno from which all of the twelve and four isolates (100%), respectively and nine out of the twenty isolates (45%) from the Gojjam samples were *Pediococcus*. None of the isolates from Wolega and Jimma samples belonged to this genus. *Streptococcus* and *Lactobacillus* constituted the second largest number of isolates. *Streptococcus* comprised of 50%, 41.67%, 15% of Jimma, Wolega and Gojjam samples, respectively. 50%, 37.5% and 20%, of the isolates from the Wolega, Jimma and Gojjam samples, respectively, were considered to be *Lactobacillus*. *Streptococcus* and *Lactobacillus*. They have not been identified from among the isolates taken from Sidamo and Sheno samples. It was only able to identify *Enterococcus* from isolates taken from Gojjam samples (20%). It might be due to faecal contamination of the samples during any of the processes; as reported by Frazier et al. (1988) describing *Enterococci* originate in the intestinal tracts of humans and animals and are sometimes used as indicator organisms of faecal contamination of foods. 25% of the Jimma and 8.3% of the Wolega isolates were of the genus *Lactococcus*.

Comparing the growth difference of the isolates from a 2week old sample and a 2 month old butter sample, it was able to notice that a diverse (Three LAB genera) type of LAB were isolated from ripened butter samples (1.5mo and 2mo) (Annex III). From Gojjam samples with 1.5 month old and Wolega samples with 2 months old, it was able to isolate three different species. However, a 2 week old Sidamo and Sheno samples gave only one type of LAB isolate, 2 weeks old Jimma and Gojjam samples managed to give two different types of isolates (Fig. 5). This increase in the number and type of species as the age of 'Qibe' increases could be due to the fact that the ripening of the butter over a time period through fermentation of carbohydrates created favorable acidic environment for the growth of the different species of lactic acid bacteria. Results obtained in this study showed that cow's butter "Qibe" contained a diversity of LAB and

diverse types of LAB isolates were identified. *Pediococcus*, *Streptococcus* and *Lactobacillus* were the predominant genus identified from this product. The microbiological study of this local product is not documented, so the results give an idea about the lactic acid flora in this traditionally produced product.

6. CONCLUSIONS AND RECOMMENDATIONS

It is clear that there are variations in the quality and safety of 'Qibe' considering differences in the origin, processing and handling of the product. To produce a microbiologically safe and chemically defined product study on the production, processing, transportation, handling and utilization of the product should be done. In addition, despite the fact that 'Qibe' is produced, transported, stored and sold under non-hygienic environment with a high possibility of contamination with desirable, pathogenic, non-pathogenic and/or spoilage bacteria, the lactic acid bacteria can reduce the risk of spoilage and pathogenesis. Training and extension work in dairy product processing and handling to the appropriate stakeholders is essential. Besides, the isolation and identification of the Lactic acid bacteria flora of butter with a potential in various technological applications is important. Results from this study show the heterogeneity of the lactic acid bacteria in the traditionally produced cow's butter. So, 56 isolates were identified; *Pediococcus*, *Streptococcus* and *Lactobacillus* (44.6%, 21.4% and 21.4%, respectively) were the predominant in this product. In this study, all the taxonomic determination of the lactic acid bacteria isolates were done by using morphological observations, physiological tests and some biochemical tests. This method is very useful and is the most widely recognized approach but the classification and identification of the isolates to the species level would be more possible if additional tests like the API strip system software analysis and molecular techniques such as the 16S RNA studies were performed. Further studies should, therefore, focus on the phenotypic characterization of the isolates by genotypic methods in addition to evaluation of the promising isolates for their important technological characteristics like antimicrobial and probiotic usage and attributes that contribute to flavor and texture development.

Therefore, based on the above conclusions, the following recommendations are given:

- The factors which resulted in predominance of the species of LAB isolated from 'Qibe' originating from different agro-ecological zones should be studied.
- The findings of this study should be confirmed by rapid and genotypic methods.
- The genera of LAB isolated should be identified to the species level.

- The technological performance of the isolates should be determined.
- As future perspective: - The possible combinations of the LAB isolates to be used in food industry, human health should be studied.

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APPENDICES

Annex 1. Chemicals for microbiological and physiological experiments

Table 1.1. Chemicals used in microbiological and physiological experiments

No	Chemicals	Code
1	MRS agar	M0303
2	MRS broth	CM0359
3	M17 agar	CM0785
4	Lactose	MW360.31
5	Citrate	M099
6	CO ₂	-
7	Hydrogen peroxide (3%)	HP00456/08
8	Potassium hydroxide (30%)	P0068111
9	Crystal violet	CI42555
10	Ammonium Oxalate	A13129
11	Iodine	AW255.81
12	Potassium iodide (KI)	KI166
13	Ethanol (95%)	E1700212
14	Phenol	P10429
15	Basic fuchsin	C142510
16	NaCl	K23166004

Annex 2. Growth media preparation recipes

2.1. MRS agar 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

64 g of the MRS powder was suspended in 1 litre of distilled water. Through a continuous agitation the powder was mixed by heating it to 50°C. The mixture was sterilized by autoclaving at 121°C for 15 minutes. Then, the sterile media is let to cool to 50°C in a water bath and dispensed on to sterile petri-dishes (~15ml per one petri dish) in a safety cabinet after a thorough decontamination of the cabinet by UV light.

2.2. MRS broth medium preparation

The MRS broth powder contains same composition as the MRS agar powder except the absence of the agar. The preparation of the medium was done according to the manufacturer's (Oxoid) instruction as follows.

52 g of the powder was added in to 1litre of distilled water and mixed thoroughly and heated to 60°C gently until completely dissolved. Then the mixture was dispensed in to sterile universal bottles (~10ml in each) in a sterile environment.

2.3. M17 agar medium composition:

Approximate Formula per 950 ml

Pancreatic Digest of Casein.....	5.0 g
Soy Peptone.....	5.0 g
Beef Extract.....	5.0 g
Yeast Extract.....	2.5 g
Ascorbic Acid.....	0.5 g
Magnesium Sulfate.....	0.25 g
Disodium- β -glycerophosphate.....	19.0 g
Agar.....	11.0 g

48.2g of the M17 powder was suspended in 950ml of distilled water. The powder is mixed by a continuous agitation heating it to a boil gently. The mixture was sterilized by autoclaving it to 121°C for 15 minutes. Then, it was cooled to 50°C and 50ml sterile lactose solution (10%w/v) was added. The mixture was then dispensed on sterile plastic petri dishes (~15ml per one petri dish) in a sterile environment.

2.4. Lactose solution 10% (w/v)

10 g of Lactose powder was dissolved in 100ml of distilled water. Then, it was sterilized by autoclaving to 121°C for 15 minutes.

Annex 3. Physiological and biochemical characteristics of the all the LAB isolates

No	LAB isolates	Citrate test +/-	Colonial morphology	Growth at different temperatures				Growth at different NaCl concentrations		Tolerance to 60°C	
				10°C	21°C	37°C	45°C	4%	6.5%	MRS agar	M17 agar
				G/NG	G/NG	G/NG	G/NG	G/NG	G/NG	D/A	D/A
1	G1-2w	-	Cocci	G	G	G	G	NG	G	D	D
2	G2-2w	-	Coco-bacilli	NG	NG	G	G	NG	NG	A	A
3	G3-2w	-	Cocci	G	G	G	G	G	NG	D	D
4	G4-2w	-	Cocci	NG	NG	G	G	NG	G	D	A
5	G5-2w	-	Cocci	G	G	G	G	NG	G	D	D
6	G6-2w	-	Cocci	G	G	G	G	G	G	A	D
7	G7-2w	-	Cocci	NG	G	G	G	NG	NG	A	A
8	G8-2w	-	Cocci	NG	NG	G	G	NG	NG	A	A
9	G1-1.5m	+	Cocci	G	G	G	G	G	G	A	A
10	G2-1.5m	-	Cocci	NG	G	G	G	G	G	A	A
11	G3-1.5m	+	Cocci	G	G	G	G	NG	G	A	A
12	G4-1.5m	-	Bacilli	G	G	G	G	G	G	A	A
13	G5-1.5m	+	Bacilli	G	G	G	G	G	G	A	A
14	G6-1.5m	+	Cocci	G	G	G	G	G	G	A	A
15	G7-1.5m	+	Bacilli	G	G	G	G	G	G	A	A
16	G8-1.5m	+	Bacilli	G	G	G	G	G	G	A	A
17	G9-1.5m	-	Cocci	G	G	G	G	NG	G	A	A
18	G10-1.5m	-	Cocci	G	G	G	G	G	G	A	A
19	G11-1.5m	+	Bacilli	G	G	G	G	G	G	A	A
20	G12-1.5m	-	Cocci	G	G	G	G	G	G	A	A
21	Sd1-2w	-	Cocci	G	G	G	G	G	G	A	A
22	Sd2-2w	-	Cocci	G	G	G	G	G	G	A	A
23	Sd3-2w	-	Cocci	G	G	G	G	G	G	A	A
24	Sd4-2w	-	Cocci	G	G	G	G	G	G	A	A
25	Sd5-2w	-	Cocci	G	G	G	G	G	G	A	A
26	Sd6-2w	-	Cocci	G	G	G	G	G	G	A	A
27	Sd7-2w	-	Cocci	G	G	G	G	G	G	A	A
28	Sd8-2w	-	Cocci	G	G	G	G	G	G	A	A
29	Sd9-2w	-	Cocci	G	G	G	G	G	G	A	A
30	Sd10-2w	-	Cocci	G	G	G	G	G	G	A	A
31	Sd11-2w	-	Cocci	G	G	G	G	G	G	A	A
32	Sd12-2w	-	Cocci	G	G	G	G	G	G	A	A
33	J1-2w	+	Coco-bacilli	G	NG	G	NG	NG	NG	D	D
34	J2-2w	+	Cocci	NG	NG	G	NG	G	NG	D	A
35	J3-2w	+	Bacilli	G	NG	G	NG	G	NG	A	A
36	J4-2w	+	Coco-bacilli	NG	NG	G	NG	NG	NG	A	A
37	J5-2w	+	Cocci	G	NG	G	NG	G	NG	D	A
38	J6-2w	+	Bacilli	G	NG	G	NG	G	NG	A	A
39	J7-2w	+	Cocci	NG	NG	G	NG	NG	NG	A	A
40	J8-2w	+	Coco-bacilli	NG	NG	G	NG	NG	NG	A	A
41	S1-2w	-	Cocci	G	G	G	NG	NG	G	D	D
42	S2-2w	-	Cocci	NG	G	G	NG	G	G	D	D
43	S3-2w	-	Cocci	NG	G	G	NG	NG	G	D	D

44	S4-2w	-	Cocci	G	G	G	NG	NG	G	D	D
45	W1-2mo	+	Bacilli	G	NG	G	NG	NG	G	A	D
46	W2-2mo	+	Bacilli	G	NG	G	NG	NG	G	A	D
47	W3-2mo	+	Cocci	NG	NG	G	NG	NG	G	A	A
48	W4-2mo	+	Coco-bacilli	G	NG	G	NG	NG	NG	D	A
49	W5-2mo	+	Cocci	NG	G	G	NG	NG	NG	D	A
50	W6-2mo	+	Cocci	NG	G	G	NG	G	NG	A	A
51	W7-2mo	+	Bacilli	G	G	G	NG	NG	G	A	A
52	W8-2mo	+	Bacilli	G	NG	G	NG	NG	NG	A	A
53	W9-2mo	+	Bacilli	G	NG	G	NG	NG	NG	A	A
54	W10-2mo	+	Cocci	NG	NG	G	NG	NG	G	A	A
55	W11-2mo	+	Coco-bacilli	NG	NG	G	NG	NG	NG	A	A
56	W12-2mo	+	Bacilli	G	NG	G	NG	NG	G	A	A

KEY: Parameters; G – growth, NG – no growth, D – dead, A – alive

Samples; G – Gojjam, Sd – Sidamo, J – Jimma, S – Sheno, W – Wolega

Age; w – weeks, mo - month

Annex 4. Cultural characteristic of the LAB isolates



Figure 4.1. Characteristic snow white colonial morphology of LAB grown on MRS and M17 agars



Figure 4.2. Yellowish yeast growth on or around the LAB colony (MRS agar)

Annex 5. Representative pictorial depiction of some of the biochemical and physiological test results.



Figure 5.1. Citrate test
Citrate positives (bluish) and negatives (whole green)



Figure 5.2. Growth at 10°C



Figure 5.3. Growth at 20°C



Figure 5.4. Growth at 45°C

N.B. Growth of the bacterium at the mentioned temperature level appreciated by observing the change in turbidity of the medium from the negative control.



Figure 5.5. Growth at 10°C

N.B. No growth observed as shown by a no change in turbidity of the sample medium from the negative control

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SIGNED DECLARATION SHEET

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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