ADDIS ABABA UNIVERSITY

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EVALUATION OF ANTAGONSTIC EFFECT OF LACTIC ACID BACTERIA ISOLATED FROM *ERGO* AGAINST SELECTED FOODBORNE PATHOGENS INOCULATED IN TO *AYIB*

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LIST OF ABBREVIATIONS

ATCC= American Type Culture Collection

CFS=Cell Free Supernatant

CFU= Colony Forming Unit

EArA1- Ergo from Aratkilo coccus

EArA3-Ergo from Aratkilo coccobacillus

EC = Enterococcus

EHNRI= Ethiopian Health and Nutrition Research Institute.

EKB3- Ergo from kebena coccus

EKU1- Ergo from Kebena mucoid

EMA2a2- Ergo from Megenagna fine rough

EMA4- Ergo from Megenagna coccus

EMA5a1- Ergo from Megenagna coccus

EMA6- Ergo from Megenagna small

EMB1a3- Ergo from Megenagna medium mucoid

EMB5- Ergo from Megenagna small small

EMB6- Ergo from Megenagna small mucoid

G=Gram

GIT= Gastro Intestinal Tract

L. =Lactobacilli

LAB = Lactic Acid Bacteria

Lc = lactococcus

MRS= De-Mann, Rogassa and Sharp

MSA= Mannitol Salt Agar

P= Pediococcus

PBS= Phosphate-Buffered Saline

Ps. = pseudomonas

PSA= Pseudomonas Isolation Agar

RSM= Reconstituted Skim Milk Medium

S. = Salmonella

Sh. = Shigella

SPSS= Stastical Package for Social Sciences

SSA= Sallmonella Shigella Agar

ABSTRACT

The antagonistic effects of mixed lactic acid bacteria cultures against foodborne pathogens (*Staphylocuccus aureus* ATCC 25923*, Shigella boydii* clinical isolate*, Pseudomonas aeruginosa* ATCC 25853) were evaluated on pasteurized *ayib* stored at room temperature. The lactic acid bacteria were tested for acid tolerance at pH 2, 2.5 and 3 for three and six hrs, bile tolerance for 24 and 48 hrs at 0.3% (W/V) bile salt concentration and antimicrobial effect on selected foodborne pathogens by co-culture assay in laboratory medium. Out of the 11 LAB isolates tested for acid tolerance 6 isolates showed survivals at pH 2.5 and pH 3 for 3 hrs and most isolates showed better tolerance to bile salt up on 24 and 48 hrs. Compared to the control (without any LAB bacteria), *Ps. aeruginosa* were inhibited by all six isolates to varying degree while, *Sh. boyidii* and *S. aureus* were inhibited by five of the isolates. Mixed LAB cultures of 10^6 cfu/g and test pathogens of 10^3 cfu/g were added together and separately in to *ayib* collected from *Shola* market and pasteurized. The effects of LAB against the foodborne pathogens were followed in *ayib* stored at ambient conditions for 9 days. The antagonistic effect of mixed LAB reduced the pathogens population by more than 2 log units on average on the 6 day. Complete elimination of the test organisms was achieved on day 5, 6, and 7 for *Ps. aruginosa, S. aureus and Sh. boyidii* respectively. The result indicated the mixed LAB cultures eliminate the test pathogen.

Key words: *Ayib*, Lactic acid bacteria, foodborne pathogens, antimicrobial effect

1. INTRODUCTION

Cheese is the general name for a group of fermented milk products produced with great diversity of flavours, textures, and forms. There are more than 1,000 varieties of cheese (Fox *et al.,* 2000). Classification of cheeses as hard, soft, semi-soft is purely arbitrary and utilitarian. The most widely accepted parameter for the categorization of cheese is its moisture content and consistency or compactness (Farkye and Vedamuthu, 2002).

Most traditional cheeses produced in local dairy plants are frequently manufactured under poor hygienic conditions with different manufacturing technologies. These lead to contamination of cheese with pathogenic microorganisms and their toxins, which can cause serious food borne problems in humans (Temelli *et al*., 2006). During the modern manufacture of cheese from pasteurized milk under inadequate hygiene conditions, *S. aureus* may contaminate heat-treated milk or curd (Arqués *et al.,* 2005). Cheese manufactured from raw milk, particularly in cases of slow or insufficient acidification of the curd, has led to staphylococcal outbreaks associated with this product (Arqués *et al.,* 2005).

Cottage cheese is a type of soft-cheese which is unripened and acid coagulated for which curd is formed by acidification of milk to or near the isoelectric pH of casein (pH 4.6), (Farkye and Vedamuthu, 2002). It has a high moisture content of 55- 80% which is very high (Farkye and Vedamuthu, 2002).

Ayib is a traditional Ethiopian cottage cheese made from sour milk after the fat is removed by churning (Almaz Gonfa *et al*., 2001). It is made from raw milk that is collected and kept at room temperature for 24 to 48 hours to sour spontaneously. The pH of sour milk is usually about 4.0 (O'Mahony, 1988 cited from Mogessie Ashenafi, 2006). In traditional *ayib* making, the milk itself may have a high initial count of microorganisms and further processing may result in increase in counts (Mogessie Ashenafi, 1994a, Cited in Mogessie Ashenafi, 2006). However, since cooking of the curd is expected to decrease the count of microorganisms, *ayib* is supposed

to have a lower microbial load after heating (Mogessie Ashenafi, 1994a, cited in Mogessie Ashenafi, 2006).

Yeasts and moulds often cause problems in a number of cheese products during storage (Mexis *et al*., 2010). Their growth on the surface of cheese is responsible for unpleasant flavour development, changes in colour, and texture or deformation of cheese packages (Mexis *et al*., 2010). Furthermore, growth of psychrotrophs such as *pseudomonads* cause spoilage, showing a slimy appearance and unpleasant odour in high pH cheeses (Mexis *et al*., 2010). *P. aeruginosa* has been recognized as an infectious agent transmitted by food and water. This organism is an opportunistic pathogen affecting primarily immunocompromised people and those suffering from cystic fibrosis (Wiedmann *et al*., 2000). A large regional outbreak of gastroenteritis caused by *Shigella* by consuming fresh cheese made from pasteurized milk was recorded (Garcia-Fulgueiras *et al*., 2002).

Chemical preservatives, such as sorbate and propionic acids, are occasionally used in cheese and their products to extend their shelf-life; however such additives may cause undesirable off-flavours (Mexis *et al*., 2010). Furthermore, consumers' growing concern over the safety of foods containing synthetic chemical preservatives, along with the economic impact of spoiled foods, have led to the investigation of alternative ‘natural’ cheese preservation technologies (Mexis *et al*., 2010).

Food borne disease and food poisoning are becoming a hot issue in the world. Both of these public health problems and the microbiological spoilage of foods can be minimized by the careful choice of raw materials, correct production, and storage stages. This necessitated monitoring microbiological loads and determining particular microbial types in dairy industry should be very important to control undesirable microorganisms in milk and dairy products which can be harmful to human health (Mostert and Jooste, 2002).

Lactic acid bacteria (LAB) are applied in food production for their useful metabolic properties. They are used as starters and as probiotics **(**Bunthof *et al*., 2001)**.** LAB is employed as starter cultures in the production of fermented foods, such as cheese, yogurts, wines, and fermented meats (Bunthof *et al*., 2001)**.**

Lactic acid bacteria (LAB) have a long history of being used as biopreservatives in food and feed storage. The general preserving ability of its fermentation end products and the antibacterial effects of LAB proteinaceous bacteriocins are well documented (Sjögren *et al*., 2003). Consequently, these are used as probiotics for protecting human health. Some members of LAB produce bacteriocins and bacteriocins-like substances which may inhibit growth of spoilage and pathogenic microorganisms (Mezaini *et al*., 2008).

Probiotics is defined as live microbial feed/food supplements, which benefit the host animal by improving the nutritional value of the food and its intestinal microbial balance, (Weese and Arroyo, 2003). Different probiotic microbial species and even different strains within a species exhibit distinctive properties that can markedly affect their survival in foods, fermentation characteristics and other probiotic properties. Among various species, *Lactobacillus acidophilus*, *L. rhamnosus*, *L. casei*, have been shown to be effective in the prophylactic management of acute diarrhoea in children by modulating their immune system (Goyal *et al.* 2008).

Hence, *Ayib* is commonly handled and packaged in unsanitary conditions, at house hold level; there is a higher possibility for its being contaminated with spoilage and food borne pathogenic organisms. This makes difficult to use the product for a longer time. Therefore this study will find good biopreservative methods to extend shelf-life of *Ayib*.

2. OBJECTIVES OF THE STUDY

2.1. General Objective

- The aim of this study is to isolate, evaluate, test and analyse the effects of enrichment of Lactic acid bacteria on the food borne pathogens in traditional Ethiopian cottage cheese, *Ayib***.**

2.2. Specific Objectives

- To isolate LAB from *Ergo*.
- To test acid and bile tolerance potential of LAB isolated from ergo.
- To evaluate inhibitory effect of acid-bile tolerant LAB, against enteropathogens by co- culture assay, in laboratory medium (ex situ).
- To evaluate the antagonistic effects of mixed LAB culture on *ayib* inoculated with pathogenic microorganisms (in situ).

3. LITRATURE REVIEWS

3.1. Spoilage of dairy products

Both bacteria and fungi are responsible for microbial spoilage of cheese, but the type of spoilage depends extremely on the characteristics of individual cheese varieties (Fernandes, 2009). The comparatively high moisture content of soft cheeses makes them easily vulnerable to microbial spoilage (Teuber, 2000).

Apart from that cheeses made from raw milk can be the main sources of cheese borne outbreaks. The main reason is that, soft cheeses do not provide sufficient hurdles (pH value, water activity) to prevent the growth of pathogenic bacteria once they have contaminated or recontaminated the cheese milk and the cheese itself (Teuber, 2000).

Dairy products are spoiled by different microbes, like psycrotrophs, sporeforming bacteria, coliforms, lactic acid bacteria, molds and yeasts. While Psychrotrophs and coliforms are the common pollutant in contaminating soft cheeses like cottage cheese (Table 1) (Ledenbach and Marshall, 2009).

The best way to ensure good shelf life of soft cheeses is to exercise severe sanitary practices throughout the manufacturing steps, and post-manufacture handling. For example, during cottage cheese manufacture, it is important that all the equipment used be properly cleaned and sanitized. Coliforms and psychrotrophic bacteria are of major concern in cottage cheese (Teuber, 2000).

3.1.1 Psychotrophs

Psychrotrophs represent a substantial number of the bacteria in raw milk, in which the major groups are *pseudomonas* and related aerobic, gram-negative, rod-shaped bacteria. Often important psychrotrophs associated with raw milk contain members of the genera *Bacillus, Micrococcus, Aerococcus,* and *Lactococcus* and of the family Enterobacteriaceae. Typically, *Pseudomonas* species contribute to 65–70% of the psychrotrophs isolated from raw milk (Farkey, and Vedamuthu, 2002).

Table 1 Dairy products and typical types of spoilage microorganisms or microbial activity

Food	spoilage microorganism or microbial activity
Pasteurized milk	Psychrotrophs, sporeformers, microbial enzymatic degradation
Butter	Psychrotrophs, enzymatic degradation
Cottage cheese	Psychrotrophs, coliforms, yeasts, molds, microbial enzymatic degradation
Yogurt, yogurt-based drinks	Yeasts
Soft, fresh cheeses	Psychrotrophs, coliforms, fungi, lactic acid bacteria, microbial enzymatic degradation
Ripened cheeses	Fungi, lactic acid bacteria, spore-forming bacteria microbial enzymatic degradation

Source: Ledenbach, and Marshall (2009)

Pseudomonas spp. is important bacterial contributor to spoilage of conventionally pasteurized fluid milk products. These organisms caused milk spoilage in two different ways. First, they produce the majority of lipolytic and proteolytic enzymes secreted into raw milk during preprocessing storage. Many of these enzymes can survive pasteurization (72°C for 15 s) and even ultra-high-temperature treatments (138°C for 2 s or 149°C for 10 s) and can thus reduce the sensory quality and shelf life of processed fluid milk products. Second, postpasteurization contamination contributes most of the microorganisms, primarily *Pseudomonas* spp., that cause spoilage of conventionally pasteurized milk during refrigerated storage (Weidmann *et al.* 2000).

Pseudomonads have abilities to grow at low temperatures (3–7◦C) and to hydrolyze and use large molecules of proteins and lipids for growth. Among the *Pseudomonas*, *P. fluorescens, P. fragi* and *P. putida* are important. Because they produce very active proteolytic and lipolytic enzymes. They cause a green or yogurt-like flavor of buttermilk and sour cream via the reduction of the the diacetyl content, thus leading to an imbalance of the diacetyl to acetaldehyde ratio (Ledenbach, and Marshall, 2009).

The heating temperatures used for cottage cheese production and pasteurization are enough for the destruction of psychotrops. Their occurrence in cottage cheese indicates post-manufacturing contamination. They cause bitterness, putrefactive and rancid odors, liquefaction of curd particles, gelatinization of curd (white, opaque curd turning translucent), slimy, mucous appearance of the curd surface, and rancidity. *Pseudomonas fluorescens* also causes discoloration because of the formation of water-soluble florescent pigments (which glow under UV light). Other *Pseudomonads* also discolor cheese surface as the casein is hydrolyzed, causing darkening or yellowing of the curd. *Pseudomonads* and other psychrotrophic gram-negative bacteria have strong diacetyl reductase enzyme, which reduces diacetyl to acetoin and other reduction products, thereby causing loss of desirable flavor (Farkey, and Vedamuthu, 2002).

The pH of cottage cheese (4.5 to 4.7) is marginally favorable for the growth of gram-negative psychrotrophic bacteria, furthermore the pH of creamed curd (5.0–5.3) being the more favorable. Psychrotrophs are the bacteria that normally limit the shelf life of cottage cheese, because its salt is insufficient to inhibit the growth of contaminating bacteria. They decrease the yield and quality of cheese curd, when their cell numbers are greater than 10^6 cfu/ml in raw milk (Ledenbach, and Marshall, 2009).

West African soft cheese, wara has a relatively short shelf-life of 2 - 3 days. It is spoiled mainly by gram-negative psychrotrophic bacteria such as *Pseudomonas* spp., *Proteus* spp. and *Aeromonas* spp. that result in unfavorable off-flavors, pigment formation or slimy curd (Kosikowski and Brown, 1973). Growth of yeasts and molds, such as *Geotrichum* spp., *Penicillium* spp., *Mucor* spp. and *Alternaria* spp. have also been implicated in the spoilage and change in flavour, texture and appearance of cottage cheese (Chen and Hotchkiss,1993).

3.1.2. Enterobacteriaceae

Like psychrotrophs, coliforms which are the member of enterobacteriaceae can also spoil buttermilk and sour cream, subsequently by producing a yogurt-like flavor through diminishing the diacetyl content. In cheese production, slow lactic acid production by starter cultures favors the growth and production of gas by coliform bacteria, with coliforms having short generation

times under such conditions. In soft, mold-ripened cheeses, the pH increases during ripening, also increases the growth potential of coliform bacteria (Ledenbach, and Marshall, 2009).

3.1.3. Spore-Forming Bacteria

Raw milk is the usual source of spore-forming bacteria in finished dairy products. The most common spore-forming bacteria found in dairy products are *Bacillus licheniformis, B. cereus, B. subtilis, B. mycoides,* and *B. megaterium.* Spores of these bacteria activated by heat used for pasteurization and germinate when they have got favourable growth temperature in the dairy product (Ledenbach and Marshall, 2009).

Clostridia also cause late blowing in cheese varities such as Goud Swiss and Cheddar by forming butyric acid from lactate. Species commonly involved are *Clostridium butyricum*, *Clostridium tyrobutyricum* and *Clostridium sporogenes*, spores of which survive pasteurization and can be present in cheese milk. Contamination of milk with these organisms is often seasonal (*C. tyrobutyricum* is more prevalent in winter), and is related to the inclusion of silage in the diet of dairy cows. A very low level of contamination may be sufficient to cause late blowing (Fernandes, 2009).

In some countries, nisin, a natural antimicrobial produced by strains of *L. lactis*, has been used successfully to control late blowing by inhibiting the growth of clostridia (Fernandes, 2009).
Spores are concentrated in cheese curd, so as few as one spore per milliliter of milk can cause gassiness in some cheeses. Spore numbers of more than 25/ml were required to produce this defect in large wheels of rindless Swiss cheese. Cheeses most often affected are Swiss, Gouda, and Edam, which have a relatively high pH and moisture content, and low salt content (Ledenbach, and Marshall, 2009).

3.1.4. Moulds

Moulds are important for both ripening and spoilage of cheese. Mould spoilage is usually unpleasant in appearance, may result in musty taints and odours, can cause liquefaction of the curd and produce mycotoxin in cheese. Moulds commonly involved in cheese spoilage include members of the genera *Penicillium*, *Aspergillus*, *Cladosporium*, *Mucor*, *Fusarium*, *Monilia* and

Alternaria. Effective hygiene is important in the control of mould spoilage in cheese, particularly in ripening rooms, and rigorous cleaning procedures are needed to prevent the accumulation of mould spores (Fernandes, 2009).

3.1.5. Yeasts

Spoilage yeasts favoured by low pH and the nutritional profile to grow in cheeses. Surface moisture, often containing lactic acid, peptides, and amino acids, favors rapid growth. Many yeasts produce alcohol and CO_2, resulting in cheese that tastes yeasty. Packages of cheese packed under vacuum or in modified atmospheres can bulge as a result of the large amount of CO_2 produced by lipolysis produces short-chain fatty acids that combine with ethanol to form fruity esters. Some proteolytic yeast strains produce sulfides, resulting in an egg odor. Common contaminating yeasts of cheeses include *Candida* spp., *Kluyveromyces marxianus*, *Geotrichum candidum*, *Debaryomyces hansenii*, and *Pichia* spp. (Ledenbach, and Marshall, 2009).

3.2. Pathogens in cheese

Listeria monocytogenes, salmonellae and enteropathogenic *Escherichia coli* (EPEC) are the most severe outbreaks linked to cheese. Pathogens initially present in the milk may be the source of contamination for cheeses made from raw milk. Pathogens may also enter cheese during processing in unsanitary condition. The characteristics of individual cheese varieties greatly influence the potential occurrence and survival of pathogens. Process and storage temperature, acid production by starter cultures and the addition of salt are all important. In general, soft and semi-soft cheeses with high water activities present fewer barriers to pathogen survival and growth than do hard cheeses. It has been indicated that *Listeria spp* is able to multiply in soft, surface ripened cheeses, such as Brie and Camembert, but is unable to grow in properly made Cheddar, although it may survive for long periods (Fernandes, 2009).

3.2.1 *Staphylococcus spp*

Contaminations of dairy products with pathogens are caused frequently due to processing, handling and unhygienic environment. Milk and milk products like cottage cheese and khoa sweets are widely consumed since ancient times and its market demand is continuous throughout the world. The occurrence of pathogenic bacteria in these dairy products can cause severe health

hazards to people. *S. aureus* is one of those bacteria that can cause minor skin infections (pimples, boils, cellulites, toxic shock syndrome, impetigo and abscesses) as well as life threatening diseases (pneumonia, meningitis, endocarditis and septicemia) (Soomro *et al.*, 2003).

Adhesion of *staphylococci* to host cell and tissue are facilitated by protein receptors such as fibronectin, fibrinogen and IgG (Todar, 2005). About 50% strains of this organism are able to produce enterotoxin associated with food poisoning (Pyne and Wood, 1974). Heating of these bacteria at normal cooking temperature may kill it but the toxins remain (Prescott *et al.*, 2002). As little as 1.0g toxin in contaminated food produces symptoms of illness. This level of the toxin production has been found with the presence of 10^5cells/g of food; this toxin cannot be denatured after boiling (Ananthanarayna *et al.*, 2001).

3.2.2 *Listeria spp*

Listeria has six species *which* can be found in the environment and contaminated foods. These are *L. monocytogenes, L. ivanovii, L. seeligeri, L. grayi, L. innocua,* and *L. welshimeri*. Two of these species, are considered to be pathogenic. *L. monocytogenes* is pathogenic to humans especially the young, elderly, and immunocompromised (Gellin and Broome, 1989), while *L. ivanovii* is pathogenic to animals with some cases of human listeriosis reported (Reissbrodt, 2002). Several reports have described the presence of *L. monocytogenes* in vegetable, dairy, and meat products, as well as in fish and seafood (Farber *et al*, 1989).

L. monocytogenes can cause food-borne listeriosis in neonates, immunocompromised hosts and pregnant women which are at great risk. Human listeriosis has non-specific flu-like symptoms (e.g. chills, fatigue, headache, and muscular and joint pain) and gastroenteritis during early stages of infection. However, without appropriate antibiotic treatment, it can develop into septicaemia, meningitis, encephalitis, and may lead to abortion and, in some cases, death (Vazquez-Boland *et al.*, 2001). Though listeriosis is relatively rare and sporadic, it is a severe disease with high fatality rate (20%-30%). Listeriosis mostly associated with soft cheeses and ready-to-eat meat-containing food products (Kaclikova *et al.,* 2001). Ability of *L.monocytogenes* to grow at temperatures ranging between 0.4^0C to 50^0C, its high tolerance for salt and its ability to initiate growth at relative low pH (5.0-5.7 at 4^0C and 4.3-5.2 at 30^0C) make the control of this

pathogen in food very difficult. A novel approach to controlling *L.monocytogenes* in food is the use of antimicrobial compounds from LAB (Vugst and Leroy, 2007).

3.2.3 *Escherichia coli*

Escherchia coli O157:H7 has develop into a pathogen of major concern for the food and dairy industries because of its ability to cause severe illness such as haemorrhagic colitis, haemolytic uremic syndrome, and thrombotic thrombocytopenic purpura , since its identification as a human pathogen in 1982 (Riley *et al*. 1983). The disease affects all age groups and the pathogen is exceptional in its severe consequence of infection, its low infectious dose and unusual acid resistance (Buchanan and Doyle, 1977). The growth of *Escherchia coli* O157in LAB-fermenting *Ergo* until 24 hrs at pH values of 5.5-4.5 could have contributed to the development of acid tolerance, which eventually resulted in survival at pH values <4.0 for more than 24 hrs. In addition, its survival for over 36 hrs when present at high levels in fermenting milk for *Ayib* making could have resulted in the development of acid tolerance (Mekonnen Tsegaye and Mogessie Ashenafi, 2005).

3.2.4 *Clostridium botulinum*

There have been a few outbreaks of botulism linked with cheese. The source of these bacteria is from straw beds, in which the cheese was ripened. Moisture content (about 60%), pH values of 5.2 and temperature abuse are suitable conditions for growth and toxin production that led to outbreak of botulism (Fernandes, 2009).

3.2.5 *Shigella spp*

The genus *Shigella* was discovered as the cause of bacillary dysentery by the Japanese microbiologist Kiyoshi Shiga in 1898. It consists of four species *Sh. dysenteriae, Sh. flexneri, Sh. boydii* and *Sh. sonnei*, all of which are regarded as human pathogens though they differ in the severity of the illness they cause (Adams and Moss, 2008). *Sh. dysenteriae* has been responsible for epidemics of severe bacillary dysentery in tropical countries but is now rarely encountered in Europe and North America where *Sh. sonnei* is more common. *Sh. sonnei* causes the mildest

illness, while that caused by *Sh. boydii* and *Sh. flexneri* is of intermediate severity (Adams and Moss, 2008).

Foodborne cases of shigellosis are regarded as uncommon though some consider the problem to be greatly underestimated. The limited range of hosts for the organism certainly suggests that it is relatively insignificant as a foodborne problem when compared with Salmonella. In foodborne cases, the source of the organism is normally a human carrier involved in preparation of the food. In areas where sewage disposal is inadequate the organism could be transferred from human faeces by flies (Adams and Moss, 2008).

3.3. Overview of cheese production

Cheese is a solid milk product which is formed by entrapment of milk fat and coagulation of casein. The removal of whey from the curd leads to hold less water content in the curd in comparison to milk. In the production of most cheeses the curd is textured, salted, shaped, and pressed into moulds before storage and curing or ripening. Soft cheese is one type of cheese with high moisture content (55 - 80%). it is grouped into two category as fresh, unripened (cottage cheese, Ricotta, Quarg, Fromage Blanc, Neufchatel, Mozzarella) and surface mould-ripened (Brie, Camembert) (Fernandes, 2009) .

Cheese is a nutrient-dense food, the precise nutritional composition of which is determined by multifactorial parameters, including the type of milk used (species, breed, stage of lactation, and fat content) and the manufacturing and ripening procedures. In general, cheese is rich in casein constituents of milk, which are retained in the curd during manufacture, and it contains relatively small amounts of the watersoluble constituents (whey proteins, lactose, and water-soluble vitamins), which partition mainly into the whey (Fox *et al*., 2000).

Cottage cheese is an acid coagulated cheese which is soft, unripened and 10% solids content, with discrete curd particles of comparatively uniform size. The major flavor compound is diacetyl, which is added in the form of starter distillate or generated by aroma-producing cultures added to the cream dressing during manufacture. When a dry curd cottage cheese covered with a creamy dressing, it is considered as creamed Cottage cheese. The specific origin of Cottage

cheese is unknown. However, as the name implies, it was produced originally in homes (cottages) but industrial Cottage cheese production began in the USA in 1916 (Farkey, 2004). The starter culture used for cottage cheese manufacturing contains primarily, the mesophilic lactic acid bacteria, *Lactococcus lactis* ssp. *cremoris* or *Lactococcus lactis* ssp. *lactis.* Starter that contains citrate positive *Lactococcus lactis* ssp. *lactis* (formerly, *Lactococciis lactis* ssp. *lactis* biovar. *diacetyluctis)* is not satisfactory for cottage cheese manufacturing because of the production of considerable amounts CO_2 which causes the curd to float (Farkye and Vedamuthu, 2002).

Ayib is a traditional Ethiopian cottage cheese which is produced by heating of buttermilk at different temperature (between 40 and 80 ^{0}C) after removal of milk fat by churning (Mogessie Ashenafi, 1992). Churning of sour milk is carried out by slowly shaking the contents of the pot until the fat is separated. The fat is then removed and the defatted milk is heated to about 50°C until a distinct curd mass forms and floats over the whey. The whey is traditionally known as aguat. Temperature, however, can be varied between 40°C and 70°C without markedly affecting product composition and yield. *ayib* comprises 79% water, 14.7% protein, 1.8% fat, 0.9% ash and 3.1% soluble milk constituents and the yield should be at least 1 kg of *ayib* from 8 liters of milk (12.5%) (O'Mahony, 1988 cited from Mogessie Ashenafi, 2006). The product is recovered when distinict curd mass is formed and floated over the whey (Mogessie Ashenafi, 1990 cited from Mogessie Ashenafi, 2006).

3.3.1 Shelf-life of cottage cheese

The shelf-life of cottage cheese as indicated by survey in USA, UK, and Canada showed that it was spoiled within 2 weeks of storage at 5-7^{0}C and was dependent on temperature (Farkey, 2004).To extend the shelf-life of these processed products, reducing microbial growth is very important (Hotchkiss *et al,* 2006).

3.4. Lactic acid bacteria

Lactic acid bacteria are a non-taxonomic group of Gram-positive, non-sporing bacteria which produce lactic acid from different sugars by fermentation. It includes species of *Lactobacillus*,

Lactococcus, *Leuconostoc* and *Pediococcus*. (*Bifidobacterium* is sometimes included). These bacteria have broad application in the food industry (bread-making, dairy products,); even if, they can also cause food spoilage. They are nutritionally fastidious and weakly proteolytic. Many activities of the lactic acid bacteria (e.g. lactose metabolism) appear to be plasmid-linked. Lactic acid bacteria very slowly grow in the presence of air and can carry out certain oxidation reactions rather than fermentation. The hydrogen peroxide formed during these oxidations has some preservative action in fermented foods (Singleton and Sainsbury, 2006).

3.4.1 Lactic acid fermentation

It is a type of fermentation, carried out by lactic acid bacteria, in which sugars (e.g. lactose, glucose, pentoses) are converted either entirely (or almost entirely) to lactic acid (homolactic fermentation) or to amixture of lactic acid and other products (heterolactic) fermentation. Lactic acid bacteria produce either L(+)-or D(−)-lactic acid, or both – the nature of the isomer(s) formed being an important taxonomic characteristic. Which isomer is produced depends on the specificity of the NAD-dependent lactate dehydrogenase (LDH) present; species which produce racemic mixtures usually possess both D- and L-specific LDHs, but in certain lactobacilli the mixture results from the combined action of L-specific LDH and lactic acid racemase (Singleton and Sainsbury, 2006)

3.4.2. Classification of starter organisms

Several microorganisms (bacteria, yeasts, molds, or combinations of these) are employed in the manufacture of cheese and other fermented milk products as primary and secondary starter culture. Lactic acid bacteria are starter cultures used to initiate lactic acid fermentation in the commercial production of dairy products; they may be supplied in freeze-dried or frozen concentrated form. The common starters used for dairy production are homolactic lactobacilli or lactococci with leuconostocs and/or strains of *Lactococcus lactis* as aroma bacteria (Singleton and Sainsbury, 2006). .

3.4.2.1 The Genus *Lactococcus*

Lc. lactis was isolated from soured milk in1873 by Lister and named *Bacterium lactis* (Latin for bacterium of milk). It was the first bacterium isolated in pure culture. In 1909, it was renamed *Streptococcus lactis* by Lohnis and placed in the genus *Streptococcus.* Lactococci are spherical or ovoid cells that occur singly, in pairs, or in chains elongated in the direction of the chain, which can sometimes cause them to be misidentified as *Leuconostoc* spp. They grow at 10^0C but not at 45^0C or in the presence of 6.5% NaCl. They are nonmotile. Motile strains of *Lc. lactis* that have the group N antigen have recently been transferred to the genus *Vagococcus. Lc. lactis* subsp. *cremoris* gives a better flavored cheese than *Lc. lactis* subsp. *lactis. Lc. lactis* subsp. *lactis* is able to grow at $4O^0$C and, in the presence of 4% NaCl, produce NH3 from arginine and ferment maltose, whereas *Lc. lactis* subsp. *cremoris* cannot (Fox et *al*., 2000).

3.4.2.2 The Genus *Pediococcus*

Pediococcus acidilnctici is the only strain of this genus used in dairy starter cultures. But, this organism had other synonyms such as *Pediococcus lindneri, Pediococcus cerevisiae,* and *Streptococcus lindneri.* The pediococci differentiated morphologically from other lactic acid bacteria by dividing alternatively in two perpendicular directions to form tetrads. The cells do not form spores or capsules, but are gram-positive cocci of uniform size, produce DL-lactate, and are nonmotile. *Pediococcus* spp. have been found in cheese, but represented only a small proportion of the total lactic acid bacteria in the product; their precise role is not fully understood (Tamine, 2002).

3.4.2.3. The Genus *Leuconostoc*

These are spherical or lenticular cells that occur in pairs and chains and are commonly found in mesophilic cultures. Therefore, they can be confused with lactococci and (heterofermentative) lactobacilli. They are capable of producing D (-) lactate, CO_2, and aroma compounds (e.g., ethanol, diacetyl and acetic acid). These organisms are normally used in multiple or mixed-strain cheese/fermented milks starter cultures that contain flavor producers. Currently, the following species of *Leuconostoc* are recognized: *Ln. lactis, Ln. citreum, Ln. pseudomes enter oides, Ln. argentinum, Ln. fallax, Ln. amelibiosum, Ln. gelidum, Ln. carnosum, Ln. mesenteroides* subsp.

mesenteroides, Ln. mesenteroides subsp. *dextranicum,* and *Ln. mesenteroides* subsp. *cremoris* (Fox et *al*., 2000).

3.4.2.4 The Genus *Streptococcus*

This microorganism is mostly used for the manufacture of cheese (Swiss and Italian varieties), yogurt, and "bio" fermented milk products in mixture with other starter cultures. Their cells are spherical or ovoid, form chains or occur in pairs, does not grow at 15^{0}C, but most strains are able to grow between 40°C and 50°C. Such streptococci are anaerobic homofermentative lactic acid, are Gram-positive, and produce L (+)-lactate, acetaldehyde, and diacetyl from lactose in milk (Tamine, 2002).

3.4.2.5 The Genus *Lactobacillus*

The lactobacilli (i.e., rod-shaped and catalase-negative species) classified into three genera: *Thermobacterium, Streptobacterium,* and *Betabacteriurn.* In the 1980s, the genus *Lactobacillus* was still divided into three main groups (I, II, and III) resembling the previous classification (Tamine, 2002). The gram-positive cell wall of lactobacilli consists of the sacculus, made up of peptidoglycan, which is decorated by lipoteichoic acids, surface proteins, and anionic and neutral polysaccharides (Delcour, 1999).

Group I, obligately homofermentative lactobacilli

The species are *L. delbrueckii* subsp. *lactis, L. delbrueckii* subsp. *bulgaricus, L. helveticus, L. kefiranofaciens, L. acidophilus, L. gasseri* and *L. johnsonii.*

Group II, facultative heterofermentative lactobacilli. Some examples are *L. casei, L. paracasei* subsp. *paracasei, L. paracasei* subsp. *tolerans, L. rhamnosus,* and *L. plantarum;* most of these organisms are used in "bio" products.

Group III, obligately heterofermentative lactobacilli

These are not important as dairy starter cultures except for Kefir production, and some examples are *L. brevis, L. fermenturn, L. kejir, L. viridescens,* and *L. reuteri* (this organism is used in "bio" fermented milks).

3.5. Mechanism of antagonism

3.5.1. Antimicrobial effect of LAB

Lactic acid bacteria (LAB) have long been used in fermentations to preserve the nutritive qualities of various foods. Production of lactic acid and reduction of pH are the main antimicrobial effect exerted by LAB (Daeschel, 1989). In addition, a variety of antimicrobial compound are produced by LAB, which can be classified as low-molecular-mass (LMM) compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione), uncharacterized compounds, and high-molecular-mass (HMM) compounds like bacteriocins (Jay, 1982). All of which can antagonize the growth of some spoilage and pathogenic bacteria in foods.

Characterization of promising strains of lactic acid bacteria, particularly those which have potential to produce inhibitory substances that are effective over a wide range of pH and temperature may result in improved safety of fermented and processed products (Fekadu Beyene *et al*., 1998). Some reports showed that lactic acid bacteria isolated from traditional dry sausage exhibited antagonistic effects against gram- positive indicator microorganisms, while gram-negative indicator microorganisms such as *Pseudomonas fluorescences* E1p-10, *Serratia liquefaciens* E1E-22, *E. coli* 54-8T and *Providencia alcalifaciens* ELE-31 are resistant (Ammor, *et al*., 2005).

The antimicrobial activity of LAB isolated from two traditional fermented beverages (Borde and Shameta) on *S. aureus, S. flexneri, Salmonella* spp. and *E. coli* O157:H7 has also been examined. All isolates, except *E. coli* O157:H7, showed an additional 3 to 4 mm zone of inhibition over the control. *Lactobacillus* isolates had the maximum inhibitory effect against the test strains, followed by *Pedicoccus, Streptococcus* and *Leuconostoc* isolates (Girum Tadesse *et al*., 2005(b).

Similarly, the work of Esayas Assefa *et al.* (2008) have revealed that, most of LAB strains isolated from Ergo, the traditional Ethiopian fermented milk were found to inhibit *S. typhi*, *S. flexnri* and *S. aureus* even if, they showed the low diameter of inhibition zones in *S. aureus*. In

contrast, *E. coli* (ATCC-25922) was the most resistant strain; it was inhibited only by two strains of LAB.

Different reports showed that most lactic acid bacteria (LAB) produce substances that inhibit pathogenic, non-pathogenic and spoilage organisms in fermenting foods and beverages (Gilliland & Speck, 1975). The antagonistic property is certified to the lowered pH, the undissociated acids and production of other primary and secondary antimicrobial metabolites produced by LAB. The metabolites produced by the fermentation process, except the volatile ones, are kept in the foods and result in growth inhibition of food spoilage or poisoning bacteria and detoxification of noxious compounds of plant origin, (Gilliland & Speck, 1975).

3.5.2. Effect of pH and temperature on antimicrobial effect of LAB

Treating the culture supernatant of LAB isolated from *Ergo*, traditional Ethiopian fermented milk at different temperature (between 30and 80^0C) do not influence the antimicrobial effect. However sterilization (121^0C for 15 min) led to complete inactivation of the inhibitory activity of the culture supernatant. Similarly the antimicrobial activity of the culture supernatant treated at varies pH (2-10) is not exaggerated, but it is significantly affected at pH=12 (Esayas Assefa *et al.*, 2008).

3.6. Preservation of cheese

Preservatives are used in cheese and their products to extend their shelf-life; however such additives may cause undesirable off-flavours (Mexis *et al*., 2010).

3.6.1. Chemical preservatives

Chemical preservatives are food additives that extend the shelf-life of food by protecting against deterioration caused by microorganisms. Some preservatives also play a role in enhancing food safety, and these are the focus of this segment. These are benzoate, sorbates , nitrates, nitrite etc. (Scurrah, 2010).

3.6.2. Probiotics

Probiotic strains are defined as live microorganisms which give a health benefit on the host, when consumed in appropriate amounts in the food. *Lactobacillus* spp. and bifidobacteria are the probiotic strains that are at present most often being investigated (FAO/WHO, 2001). Probiotic bacteria have to accomplish a number of basic technological necessities when used in commercial probiotic products. Most importantly, probiotic bacteria have to be present in sufficient numbers in the product at the date of consumption and their antagonistic effect against pathogens or health benefits after consumption have to be maintained up to that date. In addition, no adverse effects on taste and aroma of the product should be exerted by the probiotic organisms (Heller *et al.,* 2008).

3.6.2.1 Nisin

Nisin is a class I bacteriocin (lantibiotic), which has been approved by the FDA and the EU, and is used in over 48 countries (Cotter *et al.,* 2005). Nisin is used in many dairy products and high acid foods for the dual purpose of preservation and protection against pathogenic microorganisms (Delves-Broughton, 2005). Use of nisin as biological additive to yogurt to control sensitive pathogens and to extend the shelf-life has been suggested. However, such use is limited by the sensitivity of the starter culture to nisin which may impair the fermentation depending on the concentration of nisin and the strains used (Vandenbergh, *et al*., 1993).

Beginning from 1907, the health benefit of the consumption of lactic acid bacteria that were associated with fermented milk and their involvement in prolonging the life of individual was hypothesized and advocated much by Metchnikoff (Heller, 2001). The consumption of various species of lactic acid bacteria (LAB) in the form of fermented products (as live cells), freeze dried-culture (pharmaceutical in the form of tablets, capsules or granules), or as enriched health food products (in the form of liquid or powder) has been associated with different health benefits in humans. A beneficial association of microorganisms on the human host was possibly suggested by Doderlien, who proposed that lactic acid produced from sugar by vaginal bacteria inhibited the growth of pathogenic bacteria (Holzapfel *et al.,* 2001).

Yoghurt consumption that contained *Lab. acidophilus* L1 reduced serum cholesterol concentration and possibly reduced the risk of coronary heart disease (James *et al.,* 1999). The use of *Lab. casei shirota* reduced colonization levels and decreased the severity of diarrhea due to *Escherchia coli* O157:H7 in infected infant rabbits (Ogawa *et al.*, 2002). Generally, the health-related effects of the consumption of probiotics include alleviating intestinal ailments, combating lactose-intolarance, reducing serum cholesterol and cardiac diseases, reducing colon cancer and tumer, immune enhancement, enhancing vaginal/urinary tract health, reducing bowel inflammation and food allergy, and maintaining of mucosal integrity (Isolauri, 2003).

Dairy products containing living bacteria have to be cooled during storage. This applies in particular to products containing live probiotic bacteria. Cooling is necessary to guarantee high survival rates of the probiotics, and to yield sufficient stability of the product. In addition, oxygen content, redox potential, and water activity of the product have to be considered, as the target of probiotic bacteria is the intestinal tract. This may be of considerable importance for prepackaged cheese. Cooling of probiotic cheese is also necessary to reduce or inhibit the interaction of the active microorganisms with the components of the food. The degree of interaction depends on the kind and amount of carbohydrates available, degree of hydrolysis of milk proteins and the availability of essential amino acids, and composition and degree of hydrolysis of milk lipids, determining the availability of short chain fatty acids. However, the proteolytic and lipolytic properties of the probiotic bacteria may have considerable effects on taste and flavor of the product (Heller, *et al.,* 2008).

3.6.2.2 Antagonism of probiotics

According to the work of Kanmani *et al*. (2010), the probiotic strains (*Streptococcus phocae PI80, Enterococcus faecium MC13 and Carnobacterium divergens*) inhibit most gram-positive and gram- negative pathogenic strains, but these strains failed to show inhibitory activity against *E. coli* CSH57 and *E. coli* SK39.

Antagonism between bacteria is often based on the production of metabolites that inhibit or inactivate more or less specifically other related starter organisms or even unrelated bacteria. Although antagonism caused by bacteriocins, peptides, or proteins exhibiting antibiotic

properties has been described as a limiting factor for combinations of starters and probiotics, antagonism caused by other substances also has to be considered. Substances that may be involved are hydrogen peroxide, benzoic acid, biogenic amines, and finally lactic acid (Heller, *et al.,* 2008).

If probiotics are added to the cheese after fermentation, the physiological state of the probiotics may be of considerable importance for survival during ripening and storage. This state depends on:

1. The nutritional composition of the growth medium of the probiotics in relation to the nutritional composition of the cheese to which they will be added
2. Harvesting of the culture (whether in logarithmic or stationary phase)
3. Conditions leading to transition to stationary phase
4. Treatment of the probiotics during and after harvesting

Probiotic lactobacilli which have a diversity of applications are now the best alternative to treat many infectious diseases of human being (Tagg and Dierksen, 2003).These lactic acid bacteria are well known as having many properties which make them valuable to control pathogenic microorganisms. These incorporate, the ability to adhere to cell, reduce pathogenic bacteria adherents, co-aggregate, produce organic acids, hydrogen peroxide, bacteriocin and etc., be safe and nonpathogenic, which antagonize pathogenic microorganisms (Gergor, 1999).

3.6.2.3 Criteria for the selection of probiotic micro-organisms

Probiotics are living micro-organisms which exert health benefit upon ingestion in certain numbers, further than inherent basic nutrition. It may be consumed either as a food component or as a non-food preparation. probiotics such as *Lactobacillus* and *Streptococcus* have been used traditionally in fermented dairy products to promote human health by antagonizing the microbial ecology of the host, lactose intolerance, incidence of diarrhea, mucosal immune response, levels of blood cholesterol, and cancer. Indeed, a number of probiotic bacteria are now being successfully exploited commercially (*Lactobacillus rhamnosus*), *L. casei* Shirota, and *L. acidophilus* LA-1. However, many consumers, consumer organisations, and members of the scientific community remain sceptical of such products and their associated probiotic claims. As

a result, the food industry in collaboration with different stake holders such as The Lactic Acid Bacteria Industrial Platform (LABIP) and PROBDEMO (FAIR CT-96 1028) supported by European Union-funded programmes aims to promote the generation and dissemination of consensus opinions obtained through the direct interaction of research institutions and universities (Dunne *et al.,* 1999). The following criteria were set to qualify microorganisms as probiotics according to the consensus reached by the above groups.

1. Should demonstrate non-pathogenic behavior.
2. Exhibit resistance to technological processes and to gastric acid and bile.
3. Adhere to gut epithelial tissue, and briefly persist in the gastrointestinal tract.
4. Produce antimicrobial substances and modulate host immune responses.
5. Have the ability to influence metabolic activities (cholesterol assimilation, lactase activity, vitamin production) (Dunne *et al.,* 1999).

Generally, evalution of the health and nutritional properties of probiotics is determined by both *in vitro* and *in vivo* analysis. Parameters that are employed for selecting probiotics include host specificity, safeness, adsorption to gut surface and persistence in gut, antimicrobial activity, and desirable metabolic activity are usually agreed up on. But issue such as the effect of living versus nonliving probiotics or even their survival in the intestinal tract remain open (Reid *et al*, 2003; Servin, 2004).

Commonly, the *in vitro* probiotic qualities of LAB can be studied by analyzing the acid and bile tolerance, and antimicrobial activities against enteropathogens (Brizuela *et al*., 2001). The food passage time through the human stomach and that of intestine (about 90 minutes and 24hours, respectively) are also factors to be given close attention during probiotic quality analyses of LAB (Chou and Weimer, 1999).

4. MATERIALS AND METHODS

4.1 Sample Collection

Thirteen samples of *Ergo* (200ml) were collected from 5 selected sub-cities (*Megenagna, Shola, Kebena, Arat Kilo and Saris*) of addis ababa at room temperature by using sterilized bottles. After that the samples were brought to the laboratory and kept under refrigeration at 4^0c until analysis. Twenty five (25) g of each sample were mixed with sterile 225ml of peptone water (0.1% W/V) and serially diluted according to the need (Erdogrul and Erbilir, 2006).

4.2 Measurement of pH and moisture content (%) values

The pH of each *Ayib* samples was determined for nine days by blending 10 g of *ayib* sample in a stomacher with 90ml sterilized peptone water. The pH of the homogenate was then measured using the digital pH-meter (pH-016, China). The moisture content of the *Ayib* sample was determined by allowing the samples to dry to constant weight, at 35^0C with daily recording of its weight until constant reading was obtained. The difference in weight between the initial and final reading was considered as moisture content of the sample.

4.3 Isolation, counting and purification of lactic acid bacteria from *Ergo*

4.3.1 Isolation of Lactic acid bacteria

For the isolation of lactic acid bacteria a volume of 0.1 ml of appropriate dilutions (10^{-2} -10^{-5}) of *ergo* was plated on MRS (OXOID) agar plates. Inoculated plates were incubated anaerobically at 32^0 C for 24 to 48 h in Gas pak anaerobic jar (BBL).

4.3.2 Counts of Lactic acid bacteria

A volume of 0.1 ml of appropriate dilutions of *ergo* was spread-plated in duplicates on pre-dried surface of MRS (de-mann, Rogosa and sharp) agar (Oxoid) plates. The inoculated plates were incubated anaerobically, using anaerobic jars, at 30-32^0C for 48 hours. All colonies were counted as lactic acid bacteria (Girum Tadesse *et al.,* 2005)a.

4.3.3 Purification of LAB

After colony counting, 10-20 colonies were randomly picked from countable MRS plates for further identification. Colonies of LAB were transferred in to about 5ml MRS broth (Oxoid) and was purified by repeated streaking on MRS agar. Pure cultures of LAB were then streaked on slants of MRS agar, and stored at 4^0C for further characterization.

4.4 Morphological, physiological and biochemical examinations

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

4.4.1Cell Morphology

Overnight cultures were wet mounted on microscopic slide and examined under light microscope using oil immersion objectives. Cellular morphological criteria considered during examination were cell shape (regular [rods, coccoid forms, cocci] and irregular [branched, corniformis, pleomorph), cell arrangement (singles, pairs, chains, cluster, tetrads) and motility test by stab culture method (motile, immotile).

4.4.2 KOH-test (test on lipopolysaccharide)

A colony from MRS agar plate was mixed with one or two drops of 3% KOH solution using an inoculating loop on a clean microscopic slide for 10 seconds to 2minutes following the protocol given by Gregorson (1978). Colonies were considered Gram-negative when the KOH solution became viscous and threads of 0.5-2.0 cm followed the loop-open raising. Whereas, colonies were taken as Gram-positive when there was no slime and the watery solution did not follow the loop.

4.4.3. Catalase test

The formation of bubbles when young colonies were flooded with a 3% solution of hydrogen peroxide (H_2O_2) indicated the presence of catalase.

4.4.4 Cytochrome Oxidase test

This test was conducted according to the method outlined by Kovacs (1956). After flooding the young colonies with 1% N, N-dimethyl-P-phenylene diammonium chloride in distilled water in MRS agar plates, appearance of blue color on the colonies within 30 seconds to 2 minutes indicated a positive reaction. Very weak or dubious reaction that occurred after 2 minutes was ignored.

4.4.5 Physiological characterstics

Growth at 20 and 45°C in 1 week; production of acid and gas from 1 % glucose (MRS broth without beef extract) and growth in the presence of 4 and 6.5% NaCl was performed in MRS broth for 5 days.

4.4.6. Desigination of isolate

EMA2a2- Ergo from Megenagna fine rough

EMA4- Ergo from Megenagna coccus

EMB1a3- Ergo from Megenagna medium mucoid

EMA5a1- Ergo from Megenagna coccus

EKU1- Ergo from Kebena mucoid

EArA1- Ergo from Aratkilo coccus

EMA6- Ergo from Megenagna small

EArA3- Ergo from Aratkilo coccobacillus

EMB5- Ergo from Megenagna small smooth

EKB3- Ergo from Kebena coccus

EMB6- Ergo from Megenagna small mucoid

4.5 In vitro analysis of probiotics properties of LAB

The common methods for *in vitro* analysis of probiotic properties include acid and bile tolerance and antagonism against some test organisms. In this study, acid and bile tolerance and antagonism of LAB against test pathogens were conducted following the protocol given by Hyronimus *et al.* (2000).

4.5.1 Acid tolerance

Eleven (11) LAB isolates were grown in MRS broth at 37^0C overnight. A volume of 1ml of log 7 cfu/ml of each overnight grown culture was inoculated in to 10 ml of MRS broth to give initial inoculum level of log 6 cfu/ml in duplicate tubes acidified to different pH values (2.0, 2.5 and 3.0) using 3.0M HCl. Acidified broth , not inoculated with LAB culture, can serve as control. Inoculated tubes were incubated for 3 to 6 hours at 37^0C. Cells were serially diluted 10-fold in phosphate buffer (0.1 M, pH=6.2) in order to neutralize the medium acidity. A volume of 0.1ml aliquots of appropriate dilutions (10^{-1}-10^{-3}) was spreaded on duplicate pre-dried MRS plates for viable cell count. Plates were then incubated under anaerobic condition in anaerobic jar (BBL, Gas Pak Anaerobic systems), at 37^0C for 24-48 hours (Hyronimus *et al.*, 2000).

4.5.2 Bile tolerance

Acid-tolerant isolates were examined for bile-tolerance following the procedure shown in Dunne *et al*. (2001).

Each acid-tolerant LAB strain were grown overnight at 37^0C in MRS broth. Duplicate tubes of MRS broth (10ml) supplemented with 0.3% ox gall conc. (sigma Chemical Co. St Louis, Missouri, USA) were inoculated at initial inoculums level of log 6 cfu/ml, and incubated at 37^0C for 24 to 48 hours. Culture-free MRS broth without bile salt was serving as control. A volume of 0.1ml aliquots of appropriate dilutions (10^{-1}-10^{-3}) were spreaded on surface of pre-dried MRS plates for counting. Then plates were incubated under anaerobic condition in an anaerobic jar (BBL, Gas Pak Anaerobic systems) at 37^0C for 24-48 hours.

4.6 Determination of antimicrobial activity of LAB

4.6.1. Test organisms

The test organisms (*S. aureus* ATCC 25923, *Sh. boydii* clinical isolate, *Ps. aeruginosa* ATCC 25853) were obtained from Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia (EHNRI).

4.6.2 Antimicrobial effect of LAB

A LAB culture, considered acid and bile tolerant was inoculated in to 200 ml of modified buffered MRS broth in 250ml flask (MRS broth with 1% of glucose, 2% yeast extract and 2% of sodium betaglycerol phosphate) in duplicate to give a final inoculum level of log 6 cfu/ml (Moreno *et al*., 1999).This served as a control for the test pathogen. The glucose level was reduced to 1% to reduce the level of acid production and 2% sodium betaglycerol phosphate was added as a buffer to reduce the fall in pH. Any inhibition would, thus be considered as a result of factors other than acidity or reduced pH (Moreno *et al*., 1999).

Similarly, each test pathogen was separately inoculated in 200ml modified MRS broth in duplicate to give final inoculums of log 3 cfu/ml as a control. The experimental culture consisted of inoculation of LAB culture and the test pathogen together in 200ml of modified buffered MRS broth in duplicate to give a final inoculums level of log 6 cfu /ml and log 3cfu/ml for the LAB and the test pathogen, respectively. All flasks were incubated at 32^0C for 48 hours.

Samples (10ml) from each co-cultured (LAB-test pathogen) and control flask were drawn at 0, 24 and 48 hours, and processed for enumeration. Characteristic colonies were enumerated after plating 0.1ml aliquate of appropriate dilution on Mannitol Salt agar (oxoid), SS agar (oxoid) and Pseudomonads isolation agar (Difco) for *S. aureus, Sh. boydii and Ps. aeruginosa* respectively. When growth of test enteric pathogens was not detected (<log1 cfu/ml), 1 g each sample was enriched in 9 ml of nutrient broth and incubated, at 37^0C overnight. The enriched samples were streaked on different media as before. Plates were checked for the absence of characteristic colonies of target strain, and were interpreted as complete inhibition of the strain.

4.7. Enrichment of ayib with LAB

Culture of *S. aureus* ATCC 25923 was grown in nutrient broth overnight at 37^0C. The growth suspension was serially diluted in 10 ml sterile peptone water to give ca: $3x10^4$ cfu/ml. Similarly, LAB isolates were grown overnight, at 37^0C in 10 ml MRS broth (Anteneh Tesfaye, 2011).

A mixed culture of 6 LAB isolates were prepared by transferring one ml of culture broth from each pure culture into 54 ml sterile peptone water in screw-capped bottle. The mixture represented LAB mixed culture from all groups with an approximate population of 10^7 cfu/ml. This served as a stock culture (Girum Tadesse *et al*., 2005)a.

About 200g of *Ayib* bought from *shola* market was pasteurized in water bath at 80^0C for 10 minutes. The pasteurized fresh *Ayib* was then cooled to 4^0C in refrigerator. To the 200g pasteurized and cooled fresh *Ayib*, *S. aureus* ATCC 25923 was added to give final inoculum levels of 10^3cfu/g for control. Similarly, 200g of *Ayib* was inoculated with mixed culture of LAB isolates together with *S. aureus* ATCC 25923 to give final inoculum levels of 10^6 cfu/g and 10^3 cfu/g respectively.

Samples were drawn and counted at zero hour, 1st, 2nd, 3rd, to 9th days. The same procedure was applied to the other test strains (*Sh. Boydii* and *Ps. aeruginosa* ATCC 25853). When growth of test enteric pathogens was not detected (<log1 cfu/ml), 1 gm samples was enriched in 9ml of nutrient broth and incubated, at 37^0C overnight. For detection of *S. aureus, Sh. boyidii and Ps. aeruginosa* enriched samples were streaked on Mannitol Salt agar (MSA), SS agar (SSA) and Pseudomonads isolation agar (PSA), respectively. Plates were checked for the absence of characterstic colonies of target strain, and were interpreted as complete inhibition of the strain from co- culture.

4.8. Statistical analyses

Mean and standard deviation and standard error of the mean were analysed using SPSS (version 16.0, SPSS Inc, Chicago, IL, USA, 2007). Anova and LSD was performed for means comparison at ($p<0.05$) using the same program.

5. Results

In this study, a total of 60 isolates of lactic acid bacteria (LAB) were collected from13 *Ergo* (traditional Ethiopian fermented milk) samples collected in some parts of Addis Ababa. A total of 11 isolates were randomly selected for acid and bile tolerance test of which 6, 4 and one isolates were representative of rod shape, spherical and coccobacillus morphological types, respectively.

5.1. Acid and bile tolerance test

Out of the eleven LAB isolates tested for tolerance to different pH values and exposure time, only six isolates showed tolerance to the tests (Table 1). All the six isolates survived pH 3.0 and pH 2.5 up on exposure for 3 hrs, except EkU1 and EMB1a3 that failed to grow on the medium pH 2.5 (Table 1).

The isolates were found to be relatively tolerant to pH 3.0 for 3 hrs with survival rates ranging from 5% displayed by EKU1to that of survival rate of 100% tolerance shown by isolates EMA2a2 and EMB6, followed by 96% resistance by the isolate EMA6. The same isolates showed the same pattern of tolerance to pH 2.5 with a marked decrease in their survival rate of 9.9-25%, except isolate EMB6 which was found to be very sensitive to the same condition with survival rate of 1.03%. No isolate was found to be tolerant to the growth medium adjusted to pH 2.0.

The acid tolerance of the isolates was markedly reduced at 6 hrs exposure (Table 1). Only isolates EMA6 and EMB6 were found to be tolerant to pH 3.0 with survival rate of 14.38% and 22.5%; respectively. Isolates EMA2a2 and EMB5 that were found to be tolerant to a pH 3.0 at incubation time of 3 hrs failed to grow at incubation time of 6 hrs at the same growth pH.

Table 1: A Survival rate (%) of LAB isolated from *Ergo* (Ethiopian traditional fermented milk) under acidic conditions after 3 and 6 h of incubation.

Survival rate of LAB isolates in (%)

	3 hrs			6hrs		
Isolates	pH=2	pH=2.5	pH=3	pH=2	pH=2.5	PH=3
EMA2a2	_	22.00	100.00	_	_	_
EMB1a3	_	_	25.48	_	_	_
EKU1	_	_	5.00	_	_	_
EMA6	_	25.40	96.15	_	_	14.38
EMB5	_	9.90	41.48	_	_	_
EMB6	_	1.03	100.00	_	_	22.50
EMA4	_	_	_	_	_	_
EMA5a1	_	_	_	_	_	_
EArA1	_	_	_	_	_	_
EArA3	_	_	_	_	_	
EKB3	_	_	_	_	_	

With regard to bile salt tolerance test of (0.3% w/v), isolates EMA2a2 and EKU1 showed the highest survival rate (100%) followed by EMB6 and EMA6 displaying survival rates of 96.1% and 76.5% respectively up on 24 hrs of incubation (Table 2). The least survival rate of 11% was recorded from isolate EMB5.

The bile tolerance of the isolates was found to decline as the time of incubation increased to 48 hrs. The highest bile tolerance test was recorded from EKU1 with survival rate of 100%, followed by EMB6 and EMA2a2 with survival rates of 37.7% and 11.5% respectively. The least survival rate was recorded from isolate EMA6 (8.5%). The reduction in bile tolerance as a function of time was recorded by all isolates, except isolate EKU1 that was tolerant (100%) to

bile concentration at both 24 hrs and 48 hrs incubation period (Table 2). The reduction up on long incubation time was within the range of 3 fold reduction on isolate EMB6 and to 8-9 fold reduction on EMA2a2, EMB1a3 and EMA6. The isolate EMB5 was the most sensitive isolate to bile concentration of 0.3% (W/V) with reduced survival rates of 11%, and complete inhibition at 24 and 48 hrs incubation time, respectively.

Table 2: Survival rate in (%) of different LAB isolate at 0.3% (W/V) bile salt concentration.

Isolates	Bile salt tolerance in survival rate (%)	
	24hrs	48hrs
EMA2a2	100%	11.5%
EMB1a3	64.5%	9.7%
EMA6	76.5%	8.5%
EKU1	100%	100%
EMB5	11%	_
EMB6	96.1%	37.7%

5.2 Biochemical, morphological and physiological test

All the 6 acid-bile tolerant LAB isolates were gram positive, negative for catalase and cytochrome oxidase tests, and were non-motile in stab cultures. Physiologically, all the isolates grew at 4% NaCl, 20^0C, 30^0C, 37^0C and 45^0C but did not grow at 6.5% NaCl salt concentration (data not shown). Out of the six isolates, 3 isolates (50%) did not release carbon dioxide, whereas the other 3 isolates (50%) were found to release the same CO_2 (Table3). The isolates showed variation in cultural characteristics in that, all the six isolates were straight rod shaped

in morphology and white in colour, while grouped into 3 categories in size (medium, small and fine) and texture (rough, mucoid and smooth).

Table 3: Physiological, morphological and biochemical characteristics of the isolates.

Isolates	Cultural characteristics		Glucose fermentation	Production of CO_2 from glucose	Acid production from glucose	Remarks
	Size	Texture				
EMA2a2	F	r	HrF	+	+	Lactobacillus
EMB1a3	M	m	HrF	+	+	Lactobacillus
EMA6	S	s	HF	-	+	Lactobacillus
EKU1	M	m	HF	-	+	Lactobacillus
EMB5	S	s	HrF	+	+	Lactobacillus
EMB6	S	m	HF	-	+	Lactobacillus

HF=homofermentative HrF= heterofermentive += positive test -=negative test F=fine M=medium S=small m=mucoid r=rough s=smooth

5.3 Antimicrobial effect of LAB against some enteropathogens

The effect of co-culturing of LAB isolates with different test bacteria species in modified MRS media is shown in Table 4. In the control monoculture of test bacteria, the population of *Ps. aeruginosa* increased by 2 log units, *S. aureus* by 3.6 log units and *Sh. boyidii* by 2.86 log units up on 24 hrs of incubation. As incubation time increased to 48 hrs, the count of *Ps. aeruginosa* increased by 2 log units, *Sh. boyidii* by 2 log units with no change on *S. aureus* population (Table 4). Compared to the control (without any LAB bacteria), *Ps. aeruginosa* were inhibited by all six isolates to varying degree while, *Sh. boyidii* and *S. aureus* were inhibited by five of the isolates. Isolates EMA2a2 and EMB1a3 did not show inhibitiory effect on *Sh. boyidii* and *S. aureus*, respectively as compared to the control.

Very effective antagonistic effect of LAB was recorded by the isolate EMA6 which was found to completely inhibit the population of *Ps. aeruginosa* (reduced 3 log units) up on 24 hrs co-incubation, while the isolates EMA2a2, EKU1 and EMB5 were effective against *Ps. aeruginosa* (reduced 3 log units) in 48 hrs co-incubation as compared to the initial inoculated dose. The isolate EMB1a3 and EMB6 showed no inhibitory effect on the test organisms in 48 hrs. In co-culturing LAB with *Sh. boyidii* the isolate EMA6, completely inhibited the pathogens (reduced 3 log units) within 24 hrs, whereas the isolates EMB5 and EMB6 completely inhibited the organisms (reduced 3 log units) in 48 hrs. The remaining LAB isolates did not show any inhibitory effect on *Sh. boyidii* in 48 hrs co-incubation. Furthermore, the highest inhibition in co-incubating LAB isolates with *S. aureus* was recorded by EMB5 and EMA6 that drastically reduced the population by 2.6 log units and 1.7 log units respectively in 48 hrs. The other LAB isolates (EMB1a3, EMA2a2) did not show significant antagonistic effect on *S. aureus*.

Table 4. The inhibitory activity of acid and bile-tolerant LAB against *Ps. aeruginosa, Shigella boyidii* and *S. aureus* by co-culturing in laboratory medium.

	Incubation Time					
Isolates	Log cfu/ml 0hr	pH	Log cfu/ml 24 hrs	pH	Logcfu/ml 48hrs	pH
EMA6&Ps.	3.52±.04	6.23	0±.00	4.74	0±.00	4.4
EKU1&Ps	3.44±.14	6.24	1.44±.06	4.82	0±.00	4.34
EMA2a2&Ps.	3.23±.21	6.20	1.34±13	4.77	0±.00	4.35
EMB1a3&Ps.	3.54±.04	6.26	4.47±.18	4.83	3.31±.32	4.41
EMB5&Ps	3.72±.01	6.21	2.44±.14	4.66	0±.00	4.32
EMB6&Ps	3.39±.12	6.24	3.52±.09	4.67	3.79±.10	4.35
EMA6&Shi	3.29±.02	6.26	0±.00	4.77	0±.00	4.5
EKU1&Shig	3.19±.16	6.25	5.53±.30	4.87	5.63±.22	4.41
EMA2a2&Shig	3.49±.18	6.21	6.54±.09	4.72	8.58±.05	4.31
EMB1a3&Shig	3.46±.09	6.25	4.65±1.34	4.82	5.47±.05	4.42
EMB5&Shig	3.23±.07	6.22	2.24±.34	4.72	0±.00	4.4
EMB6&Shig	3.65±.03	6.20	6.31±.15	4.70	0±.00	4.38
EMA6&Staph	3.40±.11	6.26	3.30±.09	4.79	1.70±.18	4.43
EKU1&Staph	3.32±.21	6.26	3.89±.14	4.78	1.97±.10	4.46
EMA2a2&Staph	3.31±.11	6.33	3.56±.04	4.81	3.72±.36	4.42
EMB1a3&Staph	3.36±.15	6.23	6.80±.05	4.86	7.60±.25	4.43
EMB5&Staph	3.50±.18	6.23	2.60±.73	4.71	0.95±1.35	4.42
EMB6&Staph	3.50±.02	6.23	2.33±.02	4.69	2.22±.04	4.39
Ps. aeruginosa Cont	3.45±.08	6.23	5.43±05	5.30	7.43±.09	5.1
Shigella boyidii Cont	3.75±.06	6.20	6.43±.09	4.97	8.51±.27	4.42
Staphylococcus aureus Cont	3.88±13	6.23	7.49±08	5.00	7.59±03	4.98

5.4 The fate of test pathogen in mixed LAB enriched ayib

The antagonism effect of mixed LAB culture on test organisms was monitored in situ on traditional Ayib for 9 days (Table 5). The control experiment with inoculation of the test organisms without LAB showed a steady increase of the test pathogens along the incubation time. The mixed LAB cultures were found to effectively eliminate the test organisms within 5-7 days (Table 5). The mixed LAB culture were found to reduce the population of *S. aureus* and *Ps. aeruginosa* by 2 log units (3.56 -1.63 log cfu/g; 3.57-1.55 log cfu/g) at incubation period of 4 days; whereas the total elimination of *Ps. aeruginosa* and *S. aureus* by the mixed culture of LAB isolate was achieved up on 5 and 6 days of incubation time, respectively (Table 5). Furthermore the mixed LAB culture reduced the population of *Sh. boyidii* by 1 log units on the 4th day while completely inhibited the pathogens on the 7th day.

The control test organisms in monoculture *ayib* reached the maximum count on the 5th day (8.62 log units) for *S. aureus*, 6th day for *Sh. boyidii* (8.99 log units) and 6th day for *Ps. aeruginosa* (8.37 log units). In contrast to these, the control test organisms started declining in population on the 6th day for *S. aureus* and 7th day for *Sh. boyidii* and *Ps. aeruginosa* and finally reached 3.88 log units for *S. aureus*, 6.5 log units for *Sh. boyidii* and 7.39 log units for *Ps. aeruginosa* at day 9. The *S. aureus* in the monoculture was highly decreased and reached to 3.88 log units on the 9th day as compared to the two enteropathogens (Table 5). The count of LAB from co-culture samples was reached maximum (8.6 log units) on the 6th day for LAB & *S.aureus*, 6th day (8.25 log units) for LAB & *Sh. boyidii* and 5th day (8.47 log units) for LAB & *Ps. aeruginosa* (Data not shown). There is slight decrease in population of LAB starting from 6th day (1.45 log units) for LAB& *Ps. aeruginosa* and 7th day (1.05log units) for LAB& *S. aureus* and 8th day (.98 log units) for LAB& *Sh. boyidii*.

Table 5: The inhibitory effect of mixed LAB on test pathogens inoculated in to *ayib* during storage at ambient temp for 9 days.

Isolates	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
	Log Cfu/g	pH	Log cfu/g	pH	Log cfu/g	pH	Log cfu/g	pH	Log cfu/g	pH	Log cfu/g	pH	Log cfu/g	pH
LAB &S.a	3.56	4.60	4.63	4.56	4.72	4.46	1.63	4.42	1.39	4.39	_	4.35	_	4.33
S.a cont	3.13	4.61	5.29	4.58	5.41	4.58	6.55	4.58	8.62	4.60	5.63	4.61	3.39	4.60
LAB &Shig	3.59	4.61	4.50	4.54	3.24	4.44	2.29	4.43	2.54	4.41	2.26	4.39	_	4.34
Shig. cont	3.70	4.60	5.71	4.60	6.81	4.58	7.82	4.60	7.91	4.61	8.99	4.60	6.40	4.58
LAB &Ps	3.57	4.62	3.54	4.50	2.82	4.41	1.55	4.40	_	4.40	_	4.38	_	4.37
Ps. cont	3.61	4.62	4.17	4.60	6.83	4.58	7.17	4.59	5.45	4.60	8.37	4.62	7.24	4.62

6. DISCUSSIONS

All the selected isolates were identified as *lactobacillus spps* based on the morphological, physiological and cultural characteristics. They were found to be equally divided in to homofermentative and heterofermentative types (50:50). The dominance of *lactobacillus* from different fermented drink was also substantiated by similar work on borde and shamita (>50% of LAB isolates) (Girum Taddesse *et al.,* 2005)b in Ethiopia, and on yoghurt (83%) in Khartum (Ali, 2011). However, the pattern of Lactic acid fermentation on borde and Shamita was found to be different from the present work in that the heterofermentative isolates were dominant (33%) compared to the homofermentative ones (17%). Similarly, Abdullah and Osman (2010) reported that lactic acid bacteria isolated from raw cow milk, white cheese and rob in sudan were dominated in *Lactobacillus* genera (≥69.23%). Kim *et al*. (2006) have indicated that out of four lactic acid bacterial strains isolated from raw milk, three were identified as *lactobacillus casei* sgu 0020, *Lactobacillus helveticus* SGU 0011, and *lactobacillus pentosus* SGU 0010.

The six isolates were tolerant to growth media adjusted to pH 2.5 and pH 3.0, and the tolerance was reduced by more than 75% as the incubation time increased to 6 hrs (Table 1). Only two isolates EMA6 and EMB6 were relatively tolerant to low pH for long period of time. The tolerance of LAB to pH 2.5 for 3 hrs of isolates EMB6, EMB5, EMA2a2 and EMA6 showed a survival rate of 1.03%, 9.9%, 22%, and 25.4%, respectively. Similar results were obtained by Asnake Desalegn and Mogessie Ashenafi (2010) that recorded a 24% survival percentage of *lactobacillus spp* isolated from awaze, qotchqotcha and tef dough which was exposed at pH 2.5 for 3hrs. Buntin *et al*. (2008) also reported that the survival rate of 3 LAB strains isolated from marine fish (APa4, AIa1, and ARa1) at pH 2.5 for 1 hr, showed 53%, 41% and 37%.

The survival rate of isolates to pH 3 for incubation time of 3 hrs was found to vary among the isolates. Out of the eleven isolates (55%) were found to be tolerant with different survival rate (5-100%) (Table1). With respect to tolerance of LAB to pH 3 for 3 hrs, isolates EMA2a2 and EMB6 showed a survival rate of 100% both, whereas EMA6 displayed a survival rate of 96.15%. This shows that the pH survival rate (pH 3 for 3 hrs) of some of the LAB isolates in this work are comparable to the survival rate of 72%-96% by *Lactobacillus spp* isolated from yoghurt (Boke *et*

al., 2010). This is also contrary to the report of Ketema Bacha *et al*. (2009) that only 18% of the LAB isolates from a traditional Ethiopian fermented beef sausage were tolerant to pH 3 incubated for 3 hrs with survival rate of 60-100%. Howevever, the average survival percentage of LAB isolates from awaze, qotchqotcha and tef dough was 48% (Asnake Desalegn and Mogessie Ashenafi, 2010). In addition to this, moderate survival rates of lactic acid bacteria isolated from cattle feces were observed for the four strains with 11–26% after 3 hrs while, the highest survival rate at pH 3 for 3hrs showed 100% (Hyronimus *et al*., 2000). Other report revealed that four acid tolerant strains from 200 LAB isolates had showed 80% survival after exposure to pH 3 for 3 hours (Prasad *et al*., 1998 cited in Buntin *et al*., 2008).

Furthermore the exposure of these LAB isolates to pH 3 for 6hrs reduced the survivors to two isolates (EMA6 and EMB6) with survival rate of 14.38% and 22.5% respectively. This shows that the survival rate of the isolates in the present work was much reduced up on 6 hrs of incubation. Contrary to the report of Asnake Desalegn and Mogessie Ashenafi (2010) who reported a better survival rate of 38% from *lactobacillus spps.* isolated from awaze, qotchqotcha and tef dough to pH 3 for 6 hrs. In addition to this moderate survival rates of lactic acid bacteria isolated from cattle feces observed for the four strains with 0.2–15% after 6 hrs at pH 3 while, the highest survival rate were 55% (Hyronimus *et al*., 2000). Similarly, Ktema Bacha *et al*., (2009) showed out of 56 *lactobacilli* isolated from 'Wakalim', a traditional Ethiopian fermented beef sausage and exposed to pH 3 for 6 hrs, 11 isolates revealed 1-20% survival rate.

The six isolates were found to show more consistence tolerance to bile test compared to acid tolerance.The survival rate of most of these isolates up on 24 hrs incubation time was between 64.5%-100%. This is similar to the bile tolerance (47.8-100%) of different *Lactobacillus species* isolated from conventional yogurt samples recorded by Ashraf *et al*. (2009). A survival rate of more than 60% was also recorded from strains of *lactobacilli* isolated from traditional fermented food in Thai up on 24 hrs incubation (Klayraung *et al*., 2008).

In the present study, most of the isolates also showed bile tolerance up on 48 hrs incubation with a much reduced survival rate of 8.5-37.7% with the exception of EKU1 that showed a dramatic survival rate of 100%. Similarly, Buntin *et al*. (2008) reported that lactic acid bacteria isolated

from marine fish were showed 20% survival percentage up on 48 hrs incubation. While, All five strains of *L. acidophilus* obtained from the Dairy Microbiology Laboratory in Oklahoma State University exhibited excellent bile tolerance by showing ≥ 50% survival percentage up on 48 hrs incubation period(Oh *et al*., 2000).

So our acid-bile tolerant isolates of LAB can possibly tolerate the acidic environment of the stomach. In addition, the tolerance of these LAB isolates to 0.3% bile salt concentration is indicative for their possible survival in the small intestine (Gilliland *et al*., 1984). It is possible to consider our isolates as candidate for probiotics as they have showed better survival rate in *in vitro* selection criteria. So our isolates could possibly resist the hurdles in stomach and small intestine.

In this study, 4 out of 6 isolates except EMB1a3 and EMA2a2 were showed a better inhibitory effect in co-culture with test organisms in a laboratory medium during co-incubation for 48 hrs. But the best inhibitory effect against all the three test pathogens was observed by 2 of the isolates (EMA6 and EMB5). Isolate EMA6 is the only isolate that completely inhibited (reduced 3 log units) both *Ps. aeruginosa* and *Sh. boyidii* up on 24 hrs co-incubation. Furthermore, isolates EKU1, EMA2a2 and EMB5 totally eliminated *Ps.aeruginosa* up on 48 hrs. Isolates EMA6, EKU1 and EMB5 were reduced about 1 log unit *S. aureus* population up on 48 hrs co-incubation. Similarly, Pirarat *et al.* (2009) have observed that the growth of different pathogen was lower than the control after having co-incubated each pathogen with probiotic *Lactobacillus rhamnosus* for 24 hrs. However, the study of Nascimento *et al.* (2010) revealed *Ec. faecium* FAIR-E 198 did not show any significant inhibitory effect against *S. aureus* ATCC 27154 during the 48 hrs co- incubation times, the present work showed better inhibitory effect against *S. aureus*. Sung-Mee and Im (2008) have also showed that viable cell counts of *S. aureus* decreased by about 1 log unit from the initial cell counts within 24 hrs after exposure to the bacteriocin produced by *L. plantarum* KC 24. Similarly, the level of reduction in the population of *S. aureus* by probiotic bacteria was by 2.6 log units in co-culturing *S. aureus* with lactic acid bacteria in RSM medium according to (Tharmaraj and Shah, 2009). The antimicrobial effect of our mixed LAB against selected test pathogens was comparable with the above works and possible to use as probiotic culture. Furthermore, different to the present work, Guessas *et al*., (2007), reported that

the addition of the concentrated neutralized culture supernatant of *L. curvatus* strain Lc8 to a culture of *S. aureus* in broth medium reduced the *S. aureus* cells by 4 log units after 24 hrs and cells did not regrow within 48 hrs. According to Charlier *et al*. (2009) when the *S. aureus* and *Lc. lactis* co-cultured in broth medium in the ratio (*S. aureus*:*Lc. lactis*, 1/1 and 1/10), the population of *S. aureus* reduced by 4 log and 5 log units respectively. Voravuthikuncha *et al*. (2006) have showed that co-coulturing lactic acid bacteria strain L22 with *S. aureus* ATCC 25923 reduced the population of *S. aureus* by 8 log units in 24 hrs incubation.

In related to this work, Yesillik *et al*. (2011) also indicated that commercially fermented yoghurt had best antibacterial activity against *Ps. aeruginosa.* Similarly, the work of Abdelbasset and Djamila (2008) indicate, CFS from lactic acid bacteria (the strain LB44a) was inhibited *Pseudomonas aeruginosa* ATCC 27853. Incomparison to the work of Erdogrul and Erbilir (2006) that reported the weak antibacterial activity against *P. aeroginosa* by *Lactobacillus casei* and *Lactobacillus bulgaricus* isolated from various foods, this result showed good inhibitory effect against *P. aeroginosa*. However, the counts of *S. flexneri* reduced by about 1 log unit in fermenting borde co-incubated with LAB at 24 hrs (Girum Tadesse *et al*., 2005)b. Similarly, Hutt *et. al.* (2005) reported that *L. plantarm* 299v obtained from the culture collection of the University of Turku was reduced (0.6-3.2 log units) of *Sh. sonnei* ATCC 25931 up on 24 hrs co-incubation time.

Therefore the better inhibitory effect of our mixed in LAB culture against *Ps. aeruginosa* can be considered as one of the possible way to use this LAB as a biological weapons in minimizing spoilage of our traditional Ethiopian cottage cheese from psychrotrops.

In this study, the mixed LAB were completely inhibited the test pathogens within 5-7 days in *ayib* samples co-inoculated with test pathogens and stored at ambient temperature for nine days. The inhibition was highest against *Ps. aeruginosa* in reducing 3 log units within 5 days, followed by *S. aureus* by reducing 3 log units within 6 days and against *Sh. boyidii* in reducing 3 log units within 7 days. This is in agreement with the report of Anteneh Tesfaye *et al*. (2011) that indicated the mean count of the test pathogens decreased by 3 - 4 log units at day 3 and they were totally eliminated at day 6 or 7, in mixed LAB culture dipped *ayib* and kept at ambient

situation. Similarly, Ulusoy (2007) revealed that, kefir produced using freeze dried culture of, *lactobacilli*, *lactocccus* and *leuconostoc* as a starter had best antimicrobial effect against *S. aureus*. In addition, Radovanovic and Katic (2009) observed *S. aureus* with the same inoculum of 1.47 log cfu/ml multiplied more slowly in the mixed LAB culture than in the monoculture in skimed milk. Furthermore, the work of Anteneh Tesfaye *et al*., (2011) revealed that fermentation of *borde* by the mixed LAB cultures resulted in the reduction of test pathogens (*Escherichia coli*, *Salmonella typhimurium* DT104, and *Staphylococcus aureus*) to levels as low as log 1 cfu/ml at 24 hrs. Whereas in fermenting *shamita*, the average count of the test pathogens showed a slight increase at 6 hrs but decreased to log 2.02 cfu/ml at 24 hrs. Similarly, according to Girum Tadesse *et al*., (2005)b, the counts of *Sh. flexneri* and *S. aureus* co-cultured with LAB in *borde* reduced by greater than 1 log unit in 24 hrs. In addition to these, the reisolates of the probiotic additive from probiotic cheese containing *L. fermentum* strain ME-3 revealed some decrease in antagonistic activity against *Shigella sonnei* ATCC 25931, *Staphylococcus aureus* B46, as compared with the original culture of ME- 3 according to Songisepp *et al.* (2004). According to Kalavrouzioti *et al*. (2005), *Enterobacteriaceae* and coliforms, microorganisms indicative of the bacteriological quality of foods, were detected at low levels ($< 10^{2}$ cfu/g) in hard cheese produced from *L. rhamnosus LC 705 (B)* and *L. paracasei ssp. Paracasei DC 412 (C)* at 24 h due to the increasing population of LAB.

The resistance of *Sh. boyidii* to LAB isolates might be interpreted, due to the high survival of this pathogen at lowered pH (Adams and Moss, 2008). The ability of *shigella* to produce acid from glucose according to Adams and Moss (2008) may be leads for their survival at low pH. *S. flexneri* was reported to develop more tolerance to lactic acid than to other organic acids (Tetteh & Beuchat, 2001 cited in Girum Tadesse *et al.*, 2005)b.

7. CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

This study demonstrates that *ayib* enriched with probiotic lactic acid bacteria possesses antibacterial effect against *S. aureus* ATCC 25923, *Sh. boydii* and *Ps. aeruginosa* ATCC 25853 with the highest inhibition against *Ps. aeruginosa.* This indicated the possible use of these LABs with probiotic property as biopreservatives against spoilage and foodborne pathogens in Ayib.

Application of lactic acid bacteria as food preservatives to improve the keeping quality of our Ayib would significantly contribute to curb problems of food shortage at household level.

The result in this study suggests that LAB with probiotic property may be a good antimicrobial agent for food safety.

Considering the impacts of mixed cultures and longer survival of the LAB strains in the products, this study suggest that the isolates are possible good candidate starters and *Ayib* can be employed as vehicles for provisions of health promoting strains.

7.2 Recommendations

More research have to be performed related to

- The *in vivo* probiotic property of this LAB and its antagonistic effects on other fermented food.
- Sensory analysis of the product in comparison with the probiotic enriched products.
- Studying the effect of our LAB strains together with other fermenting microbial groups during the fermentation *Ayib*.
- The effect of our LAB in extending the shelf-life of *Ayib* by reducing spoilage microbes.

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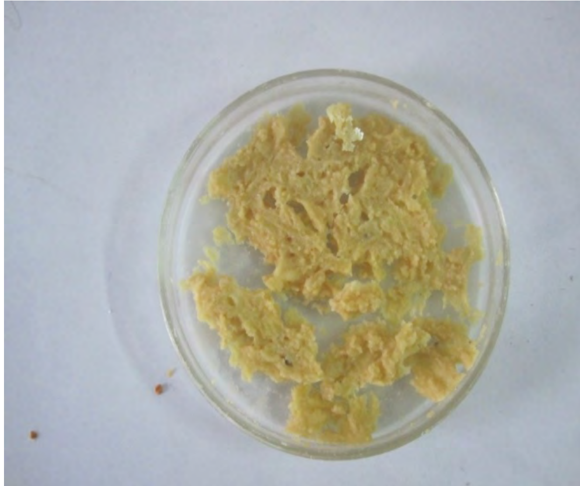
9. APPENDICES

Appendix 1. Lactic acid bacteria isolated from *Ergo* on MRS agar

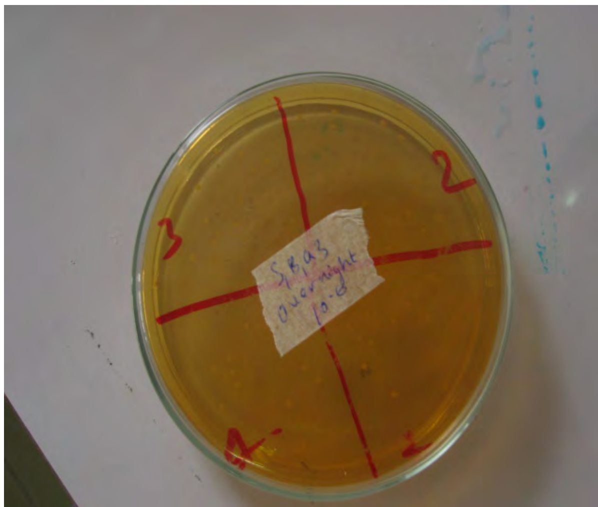
Appendix 2. Cottage cheese samples dried at $35^{0}C$ for 5 days.



a) Ayib from shoal dried

b) Ayib from shoal dried

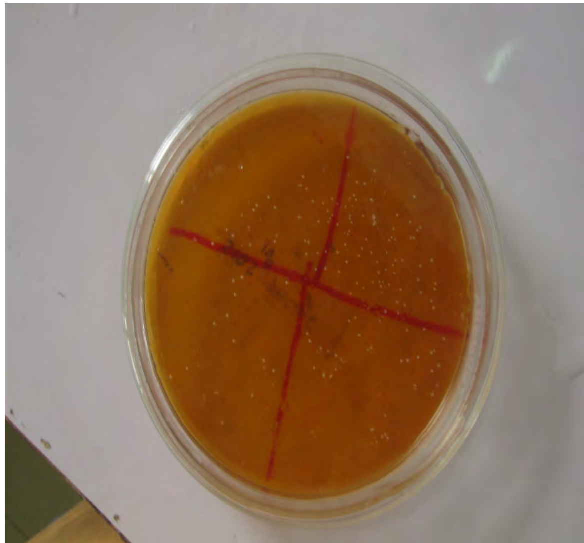
Appendix 3. Lactic acid bacteria on MRS media

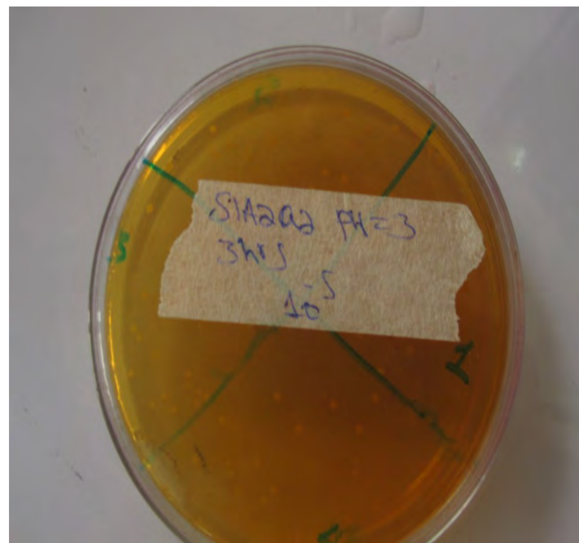


a) EMB1a3 back side

b) EMB1a3 front side

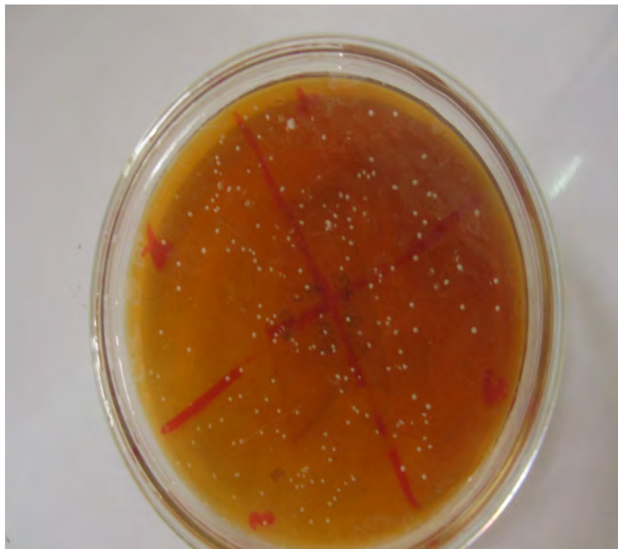
Appendix4. Acid tolerant LAB on MRS media

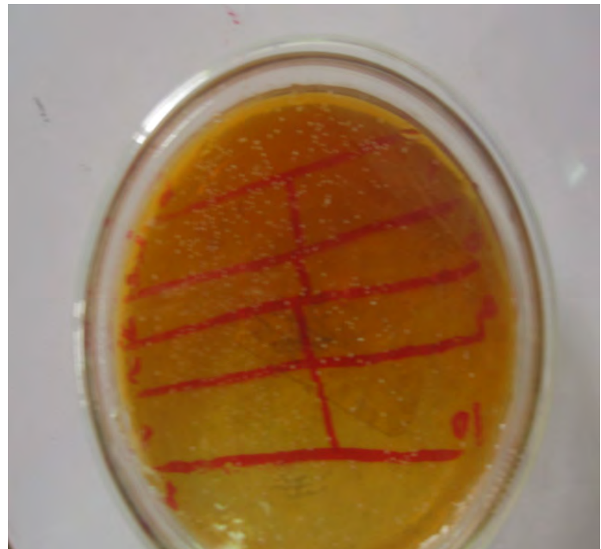




a)EMA2a2 front side b) EMA2a2 back side

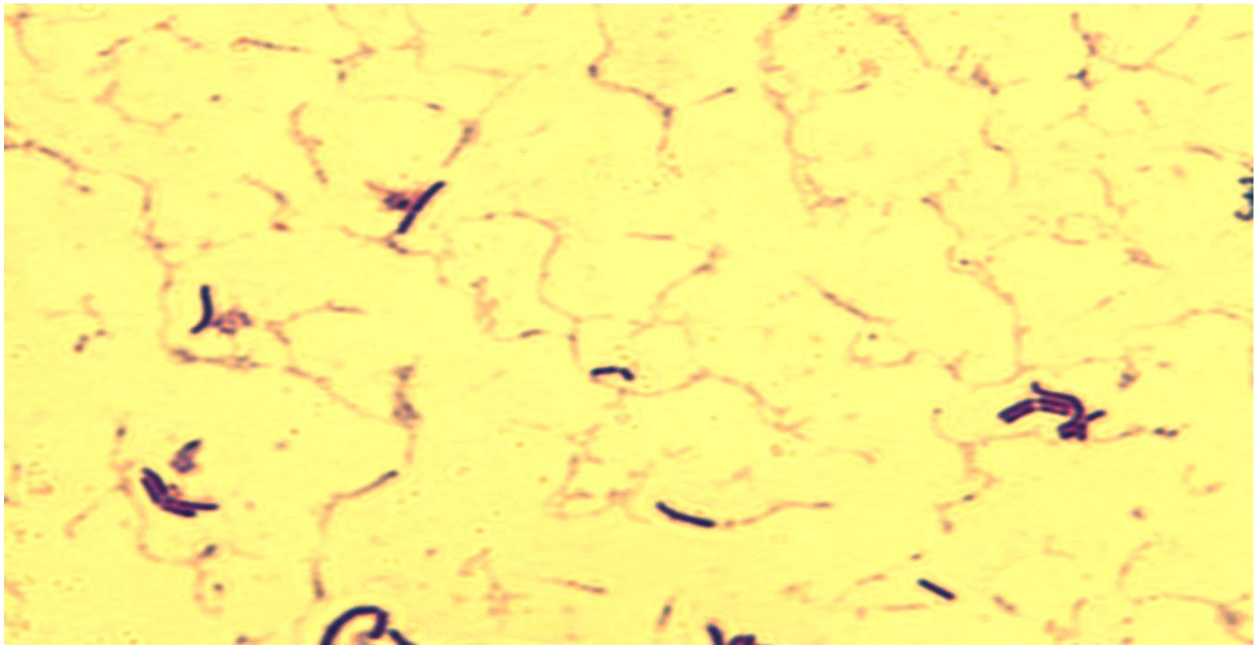
Appendix 5. Bile tolerant LAB on MRS media.



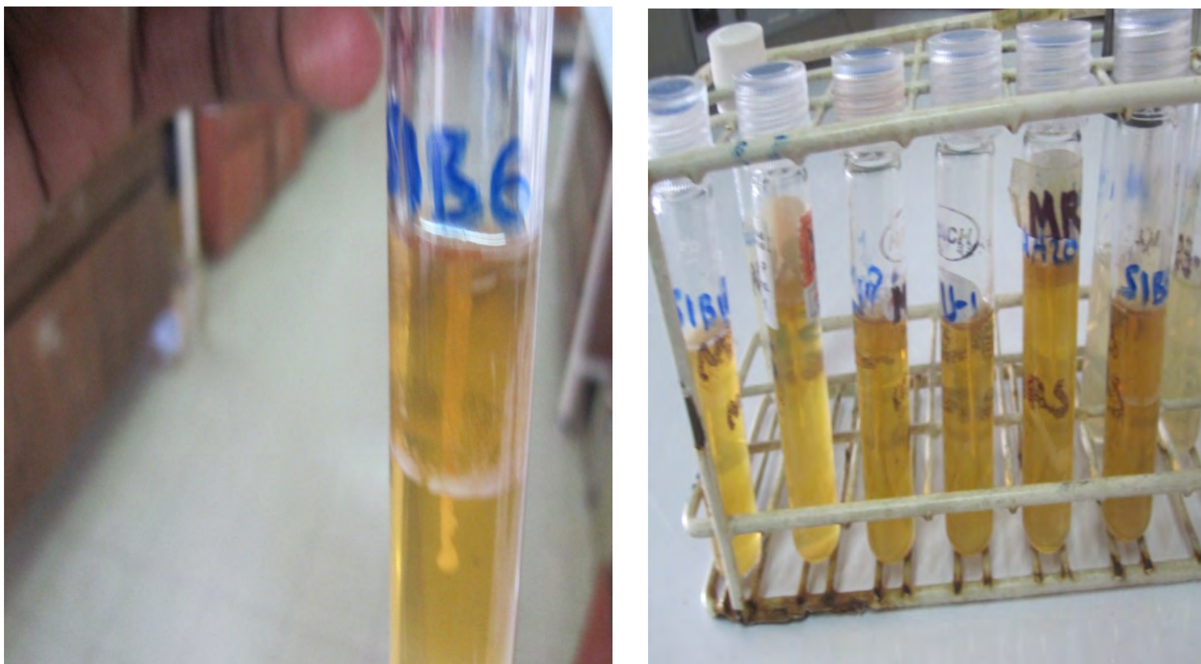


a) EMA6 front side b) EKu1 front side

Appendix 6. Microscopic image of Lactic acid bacteria by simple staining



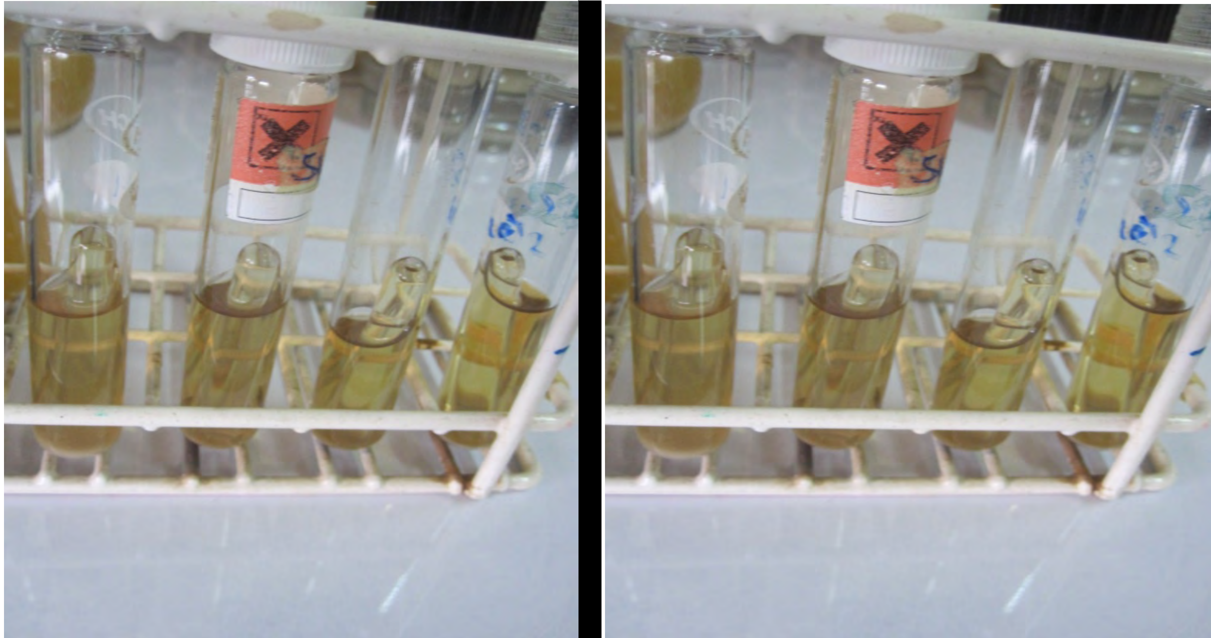
Appendix 7. Motility of LAB in semi- solid MRS media



Appendix 8. Test for antimicrobial effect of LAB in co-culture assay with foodborne pathogens.

Appendix9. Ayib enriched with mixed LAB and test pathogens

Appendix 10. Gas production of LAB in MRS broth



Appendix 11. The antagonistic effect of mixed LAB on *Shigell boyidii* and *Pseudomonas aeruginosa* as compared to control.

Appendix 12. Viable counts (log cfu/ml) of different LAB isolates at different pH for 3 hrs and 6hrs.

Acid Tolerance in mean log(cfu/ml)

	3 hrs			6 hrs		
Isolates	pH=2.0	pH=2.5	pH=3.0	pH=2	pH=2.5	pH=3
EA2a2	-	5.68±.29	5.68±.29	-	-	-
EMB1a3	-	4.67±.08	6.18±.18	-	-	-
EMA6	3.15±.22	5.51±.15	6.09±.12	-	-	5.28±.02
EKU1	-	-	5.17±.13	-	-	-
EMB5	-	5.82±.09	6.43±.18	-	-	-
EMB6	-	4.55±.01	6.65±.01	-	4.10±.02	

Appendix13: Siginificance test for the mean value of co-culture assay by LSD

Dependent Variable	(I) time= T1, T2, T3	(J) time= T1, T2, T3	Mean Difference (I-J)	Sig.
Ps. Cont	0	24	-.89082*	.008
		48	-1.00000*	.006
	24	0	.89082*	.008
		48	-.10918	.499
	48	0	1.00000*	.006
		24	.10918	.499
Shig. Cont	0	24	-2.98990*	.001
		48	-3.08136*	.001
	24	0	2.98990*	.001
		48	-.09147	.673
	48	0	3.08136*	.001
		24	.09147	.673
S.A Cont	0	24	-1.22150*	.003
		48	-1.91816*	.001
	24	0	1.22150*	.003
		48	-.69666*	.013
	48	0	1.91816*	.001
		24	.69666*	.013
EMA6& Ps	0	24	3.51771*	.000
		48	3.51771*	.000
	24	0	-3.51771*	.000
		48	.00000	1.000
	48	0	-3.51771*	.000
		24	.00000	1.000
EMA6& S.A	0	24	.10358	.490
		48	1.70580*	.001
	24	0	-.10358	.490
		48	1.60222*	.001
	48	0	-1.70580*	.001
		24	-1.60222*	.001
EMA6& Shig.	0	24	3.28989*	.000
		48	3.28989*	.000
	24	0	-3.28989*	.000
		48	.00000	1.000
	48	0	-3.28989*	.000
		24	.00000	1.000
EKu1&Ps	0	24	2.00571*	.000
		48	3.44325*	.000
	24	0	-2.00571*	.000
		48	1.43753*	.001
	48	0	-3.44325*	.000
		24	-1.43753*	.001
EKu1&S.A	0	24	-.57034*	.034
		48	1.34270*	.003
	24	0	.57034*	.034
		48	1.91304*	.001
	48	0	-1.34270*	.003
		24	-1.91304*	.001
EKu1&Shig.	0	24	-2.34119*	.002
		48	-2.44346*	.002
	24	0	2.34119*	.002
		48	-.10227	.689
	48	0	2.44346*	.002
		24	.10227	.689
EMA2a2 &S.A	0	24	-.25594	.326
		48	-.41243	.156
	24	0	.25594	.326
		48	-.15649	.526

	48	0	.41243	.156
		24	.15649	.526
EMA2a2 &Shi.	0	24	-3.05233*	.000
		48	-5.09117*	.000
	24	0	3.05233*	.000
		48	-2.03884*	.000
	48	0	5.09117*	.000
		24	2.03884*	.000
EMB1a3 &S.A	0	24	-3.44130*	.000
		48	-4.24592*	.000
	24	0	3.44130*	.000
		48	-.80462*	.017
	48	0	4.24592*	.000
		24	.80462*	.017
EMB1a3 &Shig	0	24	-1.18679	.223
		48	-2.01003	.081
	24	0	1.18679	.223
		48	-.82324	.366
	48	0	2.01003	.081
		24	.82324	.366
EMB1a3 &Ps.	0	24	-.92339*	.022
		48	.23803	.343
	24	0	.92339*	.022
		48	1.16141*	.012
	48	0	-.23803	.343
		24	-1.16141*	.012
EMB5& S.A	0	24	.90517	.384
		48	2.55019	.064
	24	0	-.90517	.384
		48	1.64502	.162
	48	0	-2.55019	.064
		24	-1.64502	.162
EMB6& S.A	0	24	1.28765*	.000
		48	1.17407*	.000
	24	0	-1.28765*	.000
		48	-.11358*	.031
	48	0	-1.17407*	.000
		24	.11358*	.031
EMB5& Shig	0	24	.98886*	.016
		48	3.22742*	.001
	24	0	-.98886*	.016
		48	2.23856*	.002
	48	0	-3.22742*	.001
		24	-2.23856*	.002
EMB6& Shig.	0	24	-2.66457*	.000
		48	3.64767*	.000
	24	0	2.66457*	.000
		48	6.31224*	.000
	48	0	-3.64767*	.000
		24	-6.31224*	.000
EMB5& Ps	0	24	1.28095*	.001
		48	3.72420*	.000
	24	0	-1.28095*	.001
		48	2.44325*	.000
	48	0	-3.72420*	.000
		24	-2.44325*	.000
EMB6& Ps.	0	24	-.13051	.302
		48	-.39893*	.032
	24	0	.13051	.302
		48	-.26841	.084
	48	0	.39893*	.032
		24	.26841	.084

*. The mean difference is significant at the 0.05 level.