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Vol. 9(29), pp. 1789-1797, 22 July, 2015
DOI: 10.5897/AJMR2015.7504
Article Number: 80B03B554662
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Evaluation of the antagonistic effect of six mixed cultures of lactic acid bacteria, isolated from the Ethiopian fermented milk *ergo*, against some foodborne pathogens inoculated into the Ethiopian cottage cheese *ayib*

Jermen Mamo Nigatu[1]*, Fassil Assefa Tuji[1] and Anteneh Tesfaye Tefera[2]

[1]Department of Microbial, Cellular and Molecular Biology, College of Natural Science, Addis Ababa University, Addis Ababa, Ethiopia.
[2]Institutes of Biotechnology, College of Natural Science, Addis Ababa University, Addis Ababa, Ethiopia.

Received 30 March, 2015; Accepted 6 July, 2015

The antagonistic effect of mixed lactic acid bacterial cultures against foodborne pathogens (*Staphylococcus aureus* ATCC 25923*, Shigella boydii* clinical isolate*, Pseudomonas aeruginosa* ATCC 25853) was evaluated in pasteurized *ayib* stored at room temperature. The lactic acid bacteria (LAB) were tested for acid tolerance at pH 2.0, 2.5, 3.0 and 3.5 for three and six h and for bile tolerance for 24 and 48 h at 0.3% (w/v) bile salt concentration. Their antimicrobial effect on selected foodborne pathogens was assessed by co-culturing assays in laboratory medium. The effect of mixed LAB cultures against the foodborne pathogens tested was followed in *ayib* stored at ambient conditions for 9 days. Only 11 LAB isolates were isolated upon their survival at a pH value of 3.5. Out of the 11 LAB isolates selected from a total of 60 based on their survival at pH 3.5, 6 isolates showed survival at pH 2.5 and pH 3.0 for 3 h with survival rates of 1.03-22% and 5-100%, respectively. The same 6 LAB isolates displayed tolerance to 0.3% bile salt concentration for up to 48 h. In the presence of acid-bile tolerant LAB isolates as compared to the control (without any LAB bacteria), *Ps. aeruginosa* was inhibited by all six to varying degree while, *Sh. boyidii* and *S. aureus* were inhibited by five of the LAB in laboratory medium. The mixed LAB culture was inoculated in to pasteurized *ayib* which was stored at ambient temperature for nine days and completely eliminated *Ps. aruginosa, S. aureus* and *Sh. boyidii* from day 5, 6, and 7, respectively. The result indicates that the mixed acid-bile tolerant LAB cultures eliminated the test pathogens in both laboratory medium and in *ayib*. The mixed acid-bile tolerant LAB culture could possibly be used as candidate potential protective starter culture for preparation of *ayib.*

Key words: *Ayib*, lactic acid bacteria, foodborne pathogens, acid-bile tolerance, inhibition.

INTRODUCTION

Cheese is the general name for a group of fermented milk products produced with great range of flavours,

textures, and forms. There are more than 1,000 varieties of cheese (Fox et al., 2000). Classification of cheeses as (Mexis et al., 2011). *Pseudomonas aeruginosa* has been recognized as an infectious agent transmitted by food hard, soft, semi-soft/semi-hard is purely arbitrary and utilitarian. The moisture content is the most widely accepted parameter for the categorization of cheese (Farkye and Vedamuthu, 2002). *Ayib* is a traditional Ethiopian cottage cheese made from sour milk after the removal of fat by churning, cooking butter milk and discarding the whey (Almaz et al., 2001). Traditionally, it is made from raw milk which is collected and kept at room temperature for 24 to 48 h to sour spontaneously. The pH of sour milk is usually about 4.0 (Mogessie, 2006). In traditional *ayib* making, the milk itself may have a high initial count of microorganisms and further processing may result in an increase in numbers (Mogessie, 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, 2006).

Many traditional cheeses produced in local dairy plants are manufactured under poor hygienic conditions with different manufacturing technologies. These lead to potential contamination of the cheese with pathogenic microorganisms and/or their toxins, which can cause serious food borne problems to humans (Temelli et al., 2006). During the manufacturing of cheese from pasteurized milk under inadequate hygiene conditions, *Staphylocuccus aureus* may contaminate heat-treated milk or curd (Ibrahim et al., 1981). Cheese manufactured from raw milk, particularly in cases of slow or insufficient acidification of the curd, has led to staphylococcal food poisoning outbreaks associated with this product (Ibrahim et al., 1981).

Ergo is a traditional Ethiopian fermented milk product which ferments spontaneously by lactic acid bacteria (Almaz et al., 2001) and from which generally *ayib* is prepared. It is thick, smooth, white color with uniform appearance that has some similarity to yoghurt. The product is semi-solid and has a pleasant odour and sour taste (Almaz et al., 2001).

Yeasts and moulds often cause problems in a cheese product during storage (Mexis et al., 2011). Their growth on the surface of the cheese is responsible for unpleasant flavour development, changes in colour, and texture or deformation of cheese packages (Mexis et al., 2011). Furthermore, growth of psychrotrophs such as *pseudomonads* causes spoilage, showing a slimy appearance and unpleasant odour in high pH cheeses (Mexis et al., 2011). Pseudomonas aeruginosa has been recognized as an infectious agent transmitted by food and water (Wiedmann et al., 2000). This organism is an opportunistic pathogen affecting primarily immune compromised people and those suffering from cystic fibrosis (Wiedmann et al., 2000). A large outbreak of gastroenteritis caused by consumption of fresh cheese made from pasteurized milk contaminated with *Shigella* was recorded in the Murcia region of Southeast Spain (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., 2011). 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., 2011).

Lactic acid bacteria (LAB) have a long history of safe use in food (Bourdichon et al., 2012), and are frequently used as bio-preservatives in food and feed storage. The general preserving ability of their fermentation end products and the antibacterial effects of LAB proteinaceous bacteriocins are well documented (Songisepp et al., 2004). Consequently, these are used as protective cultures to inactivate and reduce pathogenic bacteria, thus protecting human health (Mezaini et al., 2009).

Ayib is commonly handled and packaged in unsanitary conditions at house hold level; consequently, there is a high possibility of its being contaminated with spoilage and food borne pathogenic organisms, which reduces the shelf life of the product. Therefore, this study was aimed at finding appropriate biopreservative methods to extend the shelf-life of *ayib*.

MATERIALS AND METHODS

Sample collection

Thirteen (13) samples of *Ergo* (200 ml) were collected from 5 selected sub-cities (*Megenagna, Shola, Kebena, Arat Kilo and Saris*) of Addis Ababa using sterilized bottles. Until analyses, samples were kept under refrigeration at 4^0C in the laboratory. Twenty five (25) ml of each sample were mixed with sterile 225 ml of peptone water (0.1% W/V) to make 10^{-1} and serially diluted (Erdogrul and Erbilir, 2006). Similarly, samples of ayib were purchased from shola market, Addis Ababa, Ethiopia.

*Corresponding author. E-mail: jermenmamo@yahoo.com. Tel: +251911897595.

Abbreviations: ATCC, American type culture collection; **CFU,** colony forming unit; **LAB,** lactic acid bacteria; **MRS,** de-Man Rogossa and Sharp; **MSA,** mannitol salt agar; **NaCl,** sodium chloride; **PIA,** pseudomonas isolation agar; **SS,** *Salmonella-Shigella*; **W/V,** weight by volume; **EHNRI,** Ethiopian Health and nutrition research institute; **SPSS,** statistical package for social science; **SSA,** *Salmonella-Shigella* Agar; **LSD,** list significant difference.

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

Isolation and counting of Lactic acid bacteria

For the isolation of LAB, 0.1 ml of appropriate dilutions (10^{-2} - 10^{-5}) of *ergo* was plated in duplicate onto the surface of pre-dried de-Man Rogassa Sharp (MRS) (OXOID) agar plates. Inoculated plates were incubated anaerobically at 32°C for 24 to 48 h using a Gas pak anaerobic jar (BBL). All colonies were counted as LAB (Girum et al., 2005a).

Purification of LAB

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

Morphological, physiological and biochemical examinations

The isolates were identified according to their morphology (cell shape, cell arrangements, and motility), cultural characteristics (colony size, colony color, colony texture), physiological and biochemical characteristics (KOH-test, catalase test, cytochrome-oxidase test, growth at 20 and 45°C, production of acid and gas from 1% glucose [MRS broth without beef extract] and growth in the presence of 4 and 6.5% NaCl) based on Bergey's Manual (Nair and Surendran, 2005).

***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 foodborne organisms were done. In this study, acid and bile tolerance and antagonism of LAB against test pathogens were done (Hyronimus et al., 2000).

Acid tolerance

All the LAB isolates were grown in MRS broth at 37°C overnight. A volume of 1 ml of log 7 cfu/ml of each overnight grown culture was inoculated into 10 ml of MRS broth to give initial inoculum level of log 6 cfu/ml in duplicate tubes acidified to different pH values (2.0, 2.5, 3.0 and 3.5) using 3.0 M HCl. Inoculation of the LAB isolates to a pH 3.5 was considered as preliminary test for acid tolerance. Inoculated tubes were incubated for 3 to 6 h at 37°C. 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 0.1 ml aliquots of appropriate dilutions (10^{-1}-10^{-3}) was spread 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°C for 24-48 h (Hyronimus et al., 2009).

Bile tolerance

Acid-tolerant isolates were examined for bile-tolerance; following Dunne et al. (2001). Each acid-tolerant LAB strain was grown overnight at 37^0C in MRS broth. Duplicate tubes of MRS broth (10ml) supplemented with 0.3% bile salt conc. (sigma Chemical Co. St Louis, Missouri, USA) were inoculated at an initial inoculum level of log 6 cfu/ml, and incubated at 37°C for 24 to 48 h. A volume of 0.1 ml aliquots of appropriate dilutions (10^{-1}-10^{-3}) were spread 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°C for 24-48 h.

Determination of antimicrobial activity of LAB

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).

Antimicrobial effect of LAB

LAB isolates those that were considered acid and bile tolerant were inoculated into 200 ml of modified buffered MRS broth in 250 ml 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 LAB isolates.

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

Samples (10 ml) from each co-cultured (LAB-test pathogen) and control flask were drawn at 0, 24 and 48 h. Appropriate dilutions (0.1 ml aliquot) were plated and enumerated on Mannitol Salt agar (oxoid), Salmonella Shigella 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, incubated at 37°C overnight and streaked on the appropriate medium. Plates were checked for the presence of characteristic colonies of the target pathogenic strains.

Measurement of pH

The pH of *ayib* samples was determined by blending 10 g of *ayib* sample in a stomacher with 90 ml sterilized peptone water. The pH of the homogenate was then measured using the digital pH-meter (pH-016, Ningbo Free Trade Zone, China). *Ayib* samples were prepared for test by pasteurizing at 80°C for 10 min in order to remove vegetative cells of spoilage microorganisms.

Enrichment of ayib with LAB

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

A mixed culture of 6 LAB isolates were prepared by transferring one ml of culture broth from each pure culture into 54 ml of 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 et al., 2005a).

About 200 g of *ayib* which was bought from *shola* market and

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

Isolates	Survival rate of LAB isolates in (%)			
	3 h		6 h	
	pH=2.5	pH=3.0	pH=2.5	pH=3.0
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	-	-	-	-

No survival rate, EM: Ergo from Megenagna, EAr: Ergo from Aratkilo, EK: Ergo from Kebena while A1, A2a2, A3, A4, A6, B1, B1a3, B3, B5, B6, U1, shows different colony characteristics of the isolate during purification.

was heat treated (pasteurized) in water bath at 80°C of internal temperature for 10 min. The heat treated fresh *ayib* was then cooled to 4°C in refrigerator. To the 200 g heat treated and cooled fresh *ayib*, *S. aureus* ATCC 25923 was added to give final inoculum level of 10^3 cfu/g for the control. Similarly, 200 g of *ayib* was co-inoculated with mixed culture of LAB isolates and *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 day. The same procedure was applied to the other test strains (*Sh. boydii* and *Ps. aeruginosa* ATCC 25853) separately. When growth of test enteric pathogens was not detected (<log1 cfu/ml), a 1 g sample was enriched in 9 ml of nutrient broth and incubated at 37°C overnight. For detection of *S. aureus, Sh. boyidii* and *Ps. aeruginosa* enriched samples were streaked on Mannitol Salt agar (MSA), Salmonella Shigella agar (SSA) and Pseudomonads isolation agar (PIA), respectively. Plates were checked for the presence of characteristic colonies of the target strain.

Statistical analyses

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

RESULTS AND DISCUSSION

In this study, a total of 60 isolates of LAB were isolated from 13 *Ergo* (traditional Ethiopian fermented milk) samples collected from some parts of Addis Ababa. Eleven LAB isolates were selected from a total of 60 isolates based upon their survival at pH 3.5 (not indicated in the table). Out of the eleven LAB isolates tested for tolerance at different pH values and exposure time, only six isolates showed tolerance to the tests (Table 1).

All the 6 acid-bile tolerant LAB isolates were Gram positive, grew in 4% NaCl at 20, 30, 37 and 45°C; but did not grow at 6.5% NaCl (data not shown). Out of the six isolates, 3 isolates (50%) did not release carbon dioxide, whereas the other 3 isolates (50%) did (Table 2).

All 6 LAB isolates were identified as *Lactobacillus* spp based on the morphological, physiological and biochemical characteristics. They were found to be equally divided in to homofermentative and heterofermentative types (50:50) based on carbon dioxide release. The dominance of *Lactobacillus* from different fermented products was also substantiated by similar work on Borde and Shamita (>50% of LAB isolates) (Girum et al., 2005b) in Ethiopia, and on yoghurt (83%) in Khartum (Ali, 2011). Similarly, LAB isolated from raw cow milk, white cheese and rob in Sudan were shown to be dominated with *Lactobacillus* genera (≥69.23%) (Abdullah and Osman, 2010).

Acid tolerance test

Out of the eleven LAB isolates tested for tolerance to different pH values and exposure times, only six isolates showed tolerance to the tests (Table 2). Four isolates were tolerant to growth media adjusted to pH 2.5 for 3 h incubation period with various survival rates, and no isolates survived as the incubation time increased from 3 to 6 h (Table 2). EMB6, EMB5, EMA2a2 and EMA6 isolates showed tolerance to pH 2.5 for 3 h and survived at a rate of 1.03, 9.9, 22 and 25.4%, respectively (Table 2). None of the isolates survived to pH 2 for 3 and 6 h.

A similar study indicated a 24% survival percentage of *Lactobacillus* spp. isolated from *awaze* (fermented condiment), *qotchqotcha* (fermented condiment) and *tef*

Table 2. 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: homo fermentative, HrF: heterofermentive, +: positive test, -: negative test, F: fine, M: medium, S: small, m: mucoid, R: rough, s: smooth, EM: Ergo from Megenagna, EAr: Ergo from Aratkilo, EK: Ergo from Kebena while A2a2, A6, B1a3, B5, B6, U1, shows different colony characteristics of the isolate during purification.

Table 3. Survival rate in (%) of different LAB isolate at 0.3% (w/v) bile salt concentration.

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

No survival rate, EM: Ergo from Megenagna, EAr: Ergo from Aratkilo, EK: Ergo from Kebena while A2a2, A6, B1a3, B5, B6, U1, shows different colony characteristics of the isolate during purification.

dough which was exposed to pH 2.5 for 3 h (Asnake and Mogessie, 2010). Likewise other investigators reported that the survival rate of 3 LAB strains isolated from marine fish (APa4, AIa1, and ARa1) exposed to pH 2.5 for 1 h, showed 53, 41 and 37% survival, respectively (Buntin et al., 2008).

The survival rate of isolates at pH 3.0 for 3 h was found to vary among the isolates. Out of the eleven isolates 55% were found tolerant to pH 3.0 for 3 h with different survival rate (5-100%) (Table 2). Isolates EMA2a2 and EMB6 showed a survival rate of 100%; whereas EMA6, EMB5, EMB1a3, and EKU1 showed survival rates of 96.15, 41.48, 25.48 and 5%, respectively. Similarly, *Lactobacillus* spp isolated from yoghurt showed a survival rate between 72-96% at pH 3 for 3 h (Boke et al., 2010). But it is also contrary to the report that showed only 18% of the LAB isolates from a traditional Ethiopian fermented beef sausage were tolerant to pH 3.0 for 3 h with survival rate of 60-100% (Ketema et al., 2009).

However, the average survival percentage of LAB isolates from *awaze*, *qotchqotcha* and *tef dough* was found to be 48% (Asnake and Mogessie, 2010). In addition to this, moderate survival rate of LAB isolated from cattle feces was observed for four strains with 11-26% survival rate after 3 h with the highest survival rate at pH 3.0 for 3 h as 100% (Hyronimus et al., 2000). Other reports also showed that four acid tolerant strains from 200 LAB isolates had shown 80% survival after exposure to pH 3 for 3 h (Buntin et al., 2008).

Furthermore the exposure of these LAB isolates to pH 3 for 6 h reduced the potentially useful isolates to two isolates (EMA6 and EMB6) with survival rates of 14.38 and 22.5%, respectively. This showed that the survival rate of the isolates from the present work was reduced significantly after 6 h of incubation. In comparison to the result of the present study, it was reported that the survival rate was 38% for *Lactobacillus* spp isolated from *awaze*, *qotchqotcha* and *tef dough* at pH 3 for 6 h (Asnake and Mogessie, 2010).

In addition to this, moderate survival rates of LAB were observed for six strains with 0.2-15% after 6 h at pH 3 while, the highest survival rate was 55% (Hyronimus et al., 2000). Similarly, it was shown that out of 56 *Lactobacilli* isolated from *wakalim*, a traditional Ethiopian fermented beef sausage exposed to pH 3 for 6 h, 11 isolates showed 1-20% survival rate (Ketema et al., 2009).

Bile tolerance test

The six isolates showed more consistency in tolerance to bile (0.3% bile salt) compared to acid. This could be due to the damage of HCl to the organisms (lactobacilli) been more harmful than bile acid (Kheadr, 2006). The survival rate of most of these isolates for up on 24 h of incubation was between 64.5-100% (Table 3). This was similar to the bile tolerance (47.8-100%) of different *Lactobacillus* spp. isolated from conventional yogurt samples by Ashraf et al., (2009). A survival rate of more than 60% was also shown for strains of *Lactobacilli* isolated from traditional fermented food in Thaiup on 24 h of incubation (Klayraung et al., 2008).

In the present study, four isolates were shown to

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.

Isolate	Incubation time					
	0 h		24 h		48 h	
	Log cfu/ml	pH	Log cfu/ml	pH	Log cfu/ml	pH
EMA6&Ps	3.52±0.04	6.23	0±0.00	4.74	0±0.00	4.4
EKU1&Ps	3.44±0.14	6.24	1.44±0.06	4.82	0±0.00	4.34
EMA2a2&Ps	3.23±0.21	6.20	1.34±13	4.77	0±0.00	4.35
EMB1a3 & Ps	3.54±0.04	6.26	4.47±0.18	4.83	3.31±0.32	4.41
EMB5 & Ps	3.72±0.01	6.21	2.44±.14	4.66	0±.000	4.32
EMB6 & Ps	3.39±0.12	6.24	3.52±0.09	4.67	3.79±0.10	4.35
EMA6 & Sh	3.29±0.02	6.26	0±0.00	4.77	0±0.00	4.5
EKU1 & Sh	3.19±0.16	6.25	5.53±0.30	4.87	5.63±0.22	4.41
EMA2a2 & Sh	3.49±0.18	6.21	6.54±0.09	4.72	8.58±0.05	4.31
EMB1a3 & Sh	3.46±0.09	6.25	4.65±1.34	4.82	5.47±0.05	4.42
EMB5 & Sh	3.23±0.07	6.22	2.24±0.34	4.72	0±0.00	4.4
EMB6&Sh	3.65±0.03	6.20	6.31±0.15	4.70	0±0.00	4.38
EMA6 & Staph	3.40±0.11	6.26	3.30±0.09	4.79	1.70±0.18	4.43
EKU1 & Staph	3.32±0.21	6.26	3.89±0.14	4.78	1.97±0.10	4.46
EMA2a2 & Staph	3.31±0.11	6.33	3.56±0.04	4.81	3.72±0.36	4.42
EMB1a3 & Staph	3.36±0.15	6.23	6.80±0.05	4.86	7.60±0.25	4.43
EMB5 & Staph	3.50±0.18	6.23	2.60±0.73	4.71	0.95±1.35	4.42
EMB6 & Staph	3.50±0.02	6.23	2.33±0.02	4.69	2.22±0.04	4.39
Ps. Aeruginosa, Cont	3.45±0.08	6.23	5.43±05	5.30	7.43±0.09	5.1
Shigella boyidii, Cont	3.75±0.06	6.20	6.43±0.09	4.97	8.51±0.27	4.42
Staphylococcus aureus Cont	3.88±13	6.23	7.49±08	5.00	7.59±03	4.98

Cont: Control, Sh = *Shigella boyidii*, Staph = *Staphylococcus aureus*, Ps = *Pseudomonads aeruginosa*, EM: Ergo from Megenagna, EAr: Ergo from Aratkilo, EK: Ergo from Kebena while A2a2, A6, B1a3, B5, B6, U1, shows different colony characteristics of the isolate during purification.

tolerate bile (0.3%) up to 48 h with a survival rate of 8.5-37.7%, except isolate EKU1 that showed a dramatic survival rate of 100%. But isolate EMB5 did not survive in 0.3% bile salt concentration for 48 h. Similarly, it was reported that LAB isolated from marine fish showed 20% survival up on 48 h (Buntin et al., 2008). According to Oh et al (2000), all five strains of *L. acidophilus* exhibited excellent bile (0.3%) tolerance of ≥ 50% survival rate up on 48 h incubation period. Thus, the acid-bile tolerant LAB isolates could potentially tolerate the acidic environment of the stomach. In addition, the tolerance of these LAB isolates to 0.3% bile salt is indicative of their potential survival in the small intestine (Gilliland et al., 1984). It is possible to consider isolates as potential candidates' probiotic as they have showed reasonable survival rate during *in vitro* selection criteria. So these isolates could potentially resist the hurdles in stomach and small intestine (Dunne et al., 1999).

Antimicrobial effect of LAB against some enteropathogens

After co-culture with test organisms in a laboratory medium during co-incubation for 48 h, 4 out of 6 isolates demonstrated a better inhibitory effect on the test pathogens. But the best inhibitory effect against all the three test pathogens was observed by the isolates EMA6 and EMB5. Isolate EMA6 was the only isolate that completely inhibited (reduced 3 log units) both *Ps. aeruginosa* and *Sh. boyidii* after 24 h co-incubation. Furthermore, isolates EKU1, EMA2a2 and EMB5 totally eliminated *Ps. aeruginosa* after 48 h. Isolates EMA6, EKU1 and EMB5 were reduced about 1 log unit of *S. aureus* population up on 48 h co-incubation with LAB (Table 4). The inhibition of test pathogens by LAB in the laboratory medium might be due to the production of lactic acid, and reduction pH of the medium; and due to the production of other antibacterial substances including bacteriocin (Vuyst and Leroy, 2007).

Similarly, it was observed that the growth of different pathogen was lower than the control after co-incubating each pathogen with probiotic *Lactobacillus rhamnosus* for 24 h (Pirarat et al, 2009). However, other study revealed that *Enterococcus faecium* FAIR-E 198 did not show any significant inhibitory effect against *S. aureus* ATCC 27154 during 48 h co-culturing period, in which the present work showed better inhibitory effect against *S.*

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

Isolates	Count of test pathogens (Log cfu/g) and pH of Ayib samples													
	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
	Count	pH	Count	pH	Count	pH	Count	pH	Count	pH	Count	pH	Count	pH
LAB &Staph	3.56	4.60	4.63	4.56	4.72	4.46	1.63	4.42	1.39	4.39	-	4.35	-	4.33
Staph 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

LAB & Staph = Ayib inoculated with co-culture of *Staphylococcus aureus* with LAB, LAB & Shigella = Ayib inoculated with co-culture of *Shigella boyidii* with LAB, LAB & Ps = Ayib inoculated with co-culture of *Pseudomonas aeruginosa* with LAB, LAB=Lactic acid bacteria, Staph = *Staphylococcus aureus*, Shig = *Shigella boyidii*, Ps = *pseudomonas aeruginosa*, Cont: control, -: the microbial count is zero (totally inhibited)

aureus (Nascimento et al., 2010). In other study, 2.6 log units reduction of *S. aureus* count by probiotic bacteria was shown during co-culturing with LAB in laboratory medium (Tharmaraj and Shah, 2009). Correspondingly, when the *S. aureus* and *Lc. lactis* were co-cultured in broth medium in the ratio (*S. aureus*: *Lc. lactis*, 1/1 and 1/10), the population of *S. aureus* was reduced by 4 log and 5 log units, respectively (Charlier et al., 2009). Likewise, the co-culturing of LAB strain L22 with *S. aureus* ATCC 25923 reduced the population of *S. aureus* by 8 log units after 24 h incubation (Voravuthikunchai et al., 2006).

In comparison to the work of other investigator that reported the weak antibacterial activity against *Ps. aeroginosa* by *Lactobacillus casei* and *Lactobacillus bulgaricus* isolated from various foods, the current results showed good inhibitory effect against *Ps. aeroginosa* (Erdogrul and Erbilir, 2006). Similarly, Hutt et al., (2005) also reported that significant log units (0.6-3.2 log units) of *Sh. sonnei* ATCC 25931 was reduced up on 24 h co-incubation with *L. plantarm* 299v.

The fate of test pathogen in mixed LAB enriched *ayib*

In this study, the test pathogens were completely inhibited from *ayib* co-inoculated with mixed LAB cultures within 5-7 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 (Table 5). The inhibition of test pathogens by LAB in *ayib* enriched with mixed cultures of LAB can be as a result of the production of lactic acid, acetic acid and bacteriocin by LAB that display antibacterial activity (Vuyst and Leroy, 2007). This was in agreement with another report 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 temperature (Anteneh et al., 2011). Similarly, kefir produced using freeze dried culture of *Lactobacilli*, *Lactocccus* and *Leuconostoc* as a starter have shown the best antimicrobial effect against *S. aureus* (Ulusoy et al., 2007). Furthermore, fermentation of *borde* by 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 h (Anteneh et al., 2011). Likewise, the counts of *Sh*. *flexneri* and *S. aureus* co-cultured with LAB in *borde* reduced by greater than 1 log unit in 24 h (Girum et al., 2005b). In addition to these, the re-isolates of the probiotic additive from probiotic cheese containing *L. fermentum* strain ME-3 showed 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). *Enterobacteriaceae* and coli-forms, microorganisms' indicative of the bacteriological quality of foods, were also 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 (Kalavrouzioti et al., 2005).

The survival of *Sh. boyidii* in the presence of LAB isolates might be interpreted, due to the high resistance capacity of *shigella* at lowered pH (Adams and Moss, 2008). The ability of *Shigella* to produce acid from glucose according to Adams and Moss (2008) may be responsible for their survival at low pH. *Sh. flexneri* was reported to develop more tolerance to lactic acid than to other organic acids (Girum et al., 2005b).

Conclusions

This study demonstrated that *ayib* enriched with potential inhibitory LAB possesses antibacterial effect against *S. aureus* ATCC 25923*, Sh. boydii* and *Ps. aeruginosa* ATCC 25853 with the highest effect against *Ps. aeruginosa.* The results indicate the possible use of these LAB isolates with potential probiotic property (at least to some *in-vitro* test) as biopreservatives against spoilage

and foodborne pathogens in *ayib*.

The LAB isolates with probiotic potential not only showed to improve the safety of *ayib*, but also found extending the keeping quality of the same local fermented product. Considering the impact of mixed cultures and longer survival rate of the LAB strains in the products, this study suggests that the isolates are possible good candidate starters for preparation of *ayib*; and moreover, *ayib* can be employed as vehicles for provisions of these potential health promoting strains.

ACKNOWLEDGEMENT

The authors would like to thank the organizations that are willing to participate in this study. Moreover, we would like to acknowledge the Department of Cellular, microbial and Molecular Biology of Addis Ababa University for facilitation of laboratory space and provision of basic facilities. Addis Ababa University financed this study.

Conflict of interest

The authors declare that there is no conflict of interest among them.

REFERENCES

Abdullah SA, Osman MM (2010). Isolation and identification of lactic acid bacteria from raw cow milk, white cheese and rob in Sudan. Pak. J. Nut. 9 (12): 1203-1206.

Adams MR, Moss MO (2008). Food Microbiology. 3rd edition, RSC publishing, Cambridge, pp. 249-252.

Ali AA (2011). Isolation and identification of lactic acid bacteria isolated from traditional drinking yoghurt in Khartoum state, Sudan. C. Res. Bacteriol. 4(1): 16-22.

Almaz G, Foster HA, Holzapfel WH (2001). Field survey and literature review on traditional fermented milk products of Ethiopia. Int. J. Food Microbiol. 68: 173-186.

Anteneh T, Tetemke M, Mogessie A (2011). The inhibition of some foodborne pathogens by mixed lab cultures during preparation and storage of Ayib, a traditional Ethiopian cottage cheese. World J. Dairy & Food Sci. 6 (1): 61-66.

Anteneh T, Tetemke M, Mogessie A (2011). Antagonism of lactic acid bacteria against foodborne pathogens during fermentation and storage of bordeand shamita, traditional Ethiopian fermented beverages. Int. Food Res. J. 18(3): 1189-1194.

Ashraf M, Siddique AM, Muhammad G (2009). *In-vitro* screening of locally isolated lactobacillus species for probiotic properties. Pakistan Vet. J. 29(4): 186-190.

Asnake D, Mogessie A (2010). Evaluation of the probiotic properties and antibiotic resistance of lactic acid bacteria isolated from awaze, qotchqotch and tef dough, traditional Ethiopian fermented foods. Internet J. Food Safet. 12: 187-191.

Boke H, Aslim B, Alp G (2010). The role of resistance to bile salts and acid tolerance of exopolysaccharides (epss) produced by yogurt starter bacteria. Arch. Biol. Sci. 62 (2): 323-328.

Bourdichon F, Casaregola S, Farrokh C, Frisvad, JC, Gerds ML, Hammes WP, Harnett J, Huys G, Laulund S, Ouwehand A, Powell IB, Prajapati JB, Seto Y, Schure ET, Boven AV, Vankerckhoven V, Zgoda A, Tuijtelaars S, Hansen EB (2012). Food fermentations: microorganisms with technological beneficial use. Int. J. Food Microbiol. 154:87-97.

Buntin N, Chanthachum S, Hongpattarakere T (2008). Screening of lactic acid bacteria from gastrointestinal tracts of marine fish for their potential use as probiotics. Songklanakarin J. Sci. Technol. 30(1):141-148.

Charlier C, Cretenet M, Even S, Le Loir Y (2009). Interactions between *Staphylococcus aureus* and lactic acid bacteria: An old story with new perspectives. Int. J. Food Microbiol.131:30-39.

Dunne C, Murphy L, Flynn S, O'Mahony L, O'Halloran S, Feeney M, Morrissey D, Thornton G, Fitzgerald G, Daly C, Kiely B, Quigley EMM, Gerald C, O'Sullivan GC, Shanahan F and Collins JK (1999). Probiotics: from myth to reality: Demonstration of functionality in animal models of disease and in human clinical trials. Antonie van Leeuwenhoek. 76:279-292.

Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, Feeney M, Flynn S, Fitzgerald G, Daly C, Kiely B, O'Sullivan G, Shanahan F, Collins JK (2001). *In-vitro* selection criteria for probiotic bacteria of human origin: correlation with in-vivo findings. Am. J. Clin. Nut. 73:292-386.

Erdogrul O, Erbilir F (2006). Isolation and characterization of *Lactobacillus bulgaricus* and *Lactobacillus casei* from various foods. Turk J. Biol. 30: 39-44.

Farkye NY, Vedamuthu ER (2002). Microbiology of soft cheese. In Dairy Microbiology Handbook. 3rd edition. edited by Robinson RK. New York: John Wiley and Sons Inc. pp. 479-514.

Fox PF, Guinee TP, Cogan TM (2000). Cheese: historical aspects. In Fundamentals of Cheese Science. Gaithersburg: Aspen publishers Inc. pp. 1-9.

Garcia-Fulgueiras A, Sanchez S, Guillen JJ, Marsilla B, Alanduena A, Navaro C (2002). A large outbreak of Shigella sonne gasteroenterities associated with consumption of fresh pasteurized milk cheese. European J. Epid. 17:533-538.

Gilliland SE, Staley TE, Bush LJ (1984). Importance of bile tolerance of Lactobacillus acidophilus used as a dietary adjunct. J. Dairy Sci. 67:3045-3051.

Girum T, Eden E, Mogessie A (2005a). Assessment of the antimicrobial activity of lactic acid bacteria isolated from Borde and Shameta, traditional Ethiopian fermented beverages, on some foodborne pathogens and effect of growth medium on the inhibitory activity. Int. J. Food Safet. 5:13-20.

Girum T, Mogessie A, Eden E (2005b). Survival of E. coli O157:H7 Staphylococcus aureus, Shigella flexneri and Salmonella spp. in fermenting Borde, a traditional Ethiopian beverage. Food Cont. 16:189-196.

Hutt P, Shchepetova J, Lõivukene K, Kullisaar T. Mikelsaar M (2005). Antagonstic activity of probiotic *Lactobacilli* and *Bifidobacteria* against entero and uropathogens. J. App Microbiol. 100:1324-1332.

Hyronimus B, Le Marrec C, HadjSassi A, Deschamps A (2000). Acid and bile tolerance of spore-forming lactic acid bacteria. Int. J. Food Microbiol. 61: 193-197.

Ibrahim GF, Baldock AK, Radford DR, Ireland LB (1981). Inhibition of *Staphylococcus aureus* growth and enterotoxin-A production in cheddar cheese produced with variable starter activity. J. Food Protoc. 44: 263-267.

Kalavrouzioti I, Hatzikamari M, Litopoulou-Tzanetaki E, Tzanetakis N (2005).Production of hard cheese from caprine milk by the use of two types of probiotic culturesas adjuncts. Int. J. DairyTechnol.58: 32.

Ketema B, Tetemke M, Mogessie A (2009). In-vitro probiotic potential of lactic acid bacteria isolated from 'Wakalim', a traditional Ethiopian fermented beef sausage. Ethiopian J. H. Sci. 19(1): 21-29.

Kheadr EE(2006). Impact of acid and oxgall on antibiotic susceptibility of probiotic *Lactobacilli.* Afr. J. Agric. Res. 1 (5):172-181.

Klayraung S, Viernstein H, Sirithunyalug J, Okonogi S (2008). Probiotic properties of *Lactobacilli* isolated from thai traditional food. Sci. Pharm. 76:485-503.

Mexis SF, Chouliara E, Kontominas MG (2011). Quality Evaluation of Grated Graviera Cheese Stored at 4 and 12°C using Active and Modified Atmosphere Packaging. Pack. Technol. Sci. 24:15-29

Mezaini A, Chihib NE, Bouras AD, Nedjar-Arroume N, Hornez JP (2009). Antibacterial Activity of Some lactic acid bacteria isolated from an Algerian dairy product. J. Env. P. H. 9: 61.

Mogessie A (2006). A review on the microbiology of indigenous fermented foods and beverages of Ethiopia. Ethiopian J. Biol. Sci.

5(2):189-245.
Moreno I, Lerayer AL, de Freitas Leitao MF (1999). Detection and characterization of bacteriocin-producing *Lactococcus lactis* strains. Rev. Microbiol. 30:34-43.
Nair PS, Surendran PK (2005). Biochemical characterization of lactic acid bacteria isolated from fish and prawn. J. C. Coll. 4:48-52.
Nascimento M, Moreno1 I, YoshiteruKuaye A (2010). Antimicrobial activity of *Enterococcus faecium* fair-e 198 against Gram-positive pathogens. Braz. J. Microbiol. 41:74-81.
Oh S, Kim SH, Worobo RW (2000). Characterization and purification of a bacteriocin produced by a potential probiotic culture, *Lactobacillus acidophilus* 30SC. J. D. Sci. 83:2747-2752.
Pirarat N, Pinpimai K, Chankow K, Malila K, Chansue N, WareeNiyomtham W, ChannarongRodkhum C(2009). In Vitro efficacy of human-derived probiotic, *Lactobacillus rhamnosus* against pathogenic bacteria in fish and frogs. Thai. J. Vet. Med. 39(4):305-310.
Songisepp E, Kullisaar T, Hu¨tt P, Elias P, Brilene T, Zilmer M, Mikelsaar M (2004). A new probiotic cheese with antioxidative and antimicrobial activity. J. Dairy Sci. 87: 2017-2023.
Temelli S, Anar S, Sen C, Akyuva P (2006). Determination of microbiological contamination sources during Turkish white cheese production. F. Contam. 17:856-861.
Tharmaraj N, Shah NP (2009). Antimicrobial effects of probiotics against selected pathogenic and spoilage bacteria in cheese-based dips. Int. Food Res. J. 16:261-276.
Ulusoy BH, Çolak H, Hampikyan H, Erkan ME (2007). An in vitro study on the antibacterial effect of kefir against some food-borne pathogens. Türk. Mikrobiyol. Cem. Derg. 37 (2): 103-107.
Voravuthikunchai SP, Bilasoi S, Supamala O (2006). Antagonistic activity against pathogenic bacteria by human vaginal *Lactobacilli*. Anaerobe. 12:221-226.
Vuyst LD, Leroy F (2007). Bacteriocins from Lactic Acid Bacteria: production, purification, and food applications. J. Mol. Microbiol. Biotechnol.13:194-199.
Wiedmann M. Weilmeier D, Dineen SS, Ralyea R, Kathryn J, Boor KJ (2000). Molecular and phenotypic characterization of Pseudomonas spp. isolated from milk. Appl. Environ. Microbiol. 66:52.