ORIGINAL ARTICLE

Evaluation of Functional Properties of Lactic Acid Bacteria Isolated from raw cow milk and fermented milk 'ergo' in Ethiopia

Betemariam Kebede[1]*, Tefera Tadesse, Daniel Yimer[1], Tirsit Tibebu[1], Mulatu Workie[1], Sewunet Abera[1], Adaba Tilahun[1], Melaku Alemu[2], [1]Tadessa Daba, Adane Eshetu[1], Asab Alemneh[1], Birhanu Babiye[1], Gudeta Dida[1], Tariku Abena[1]

[1]Ethiopian institute of agricultural research, National Agricultural Biotechnology Researcher Center, Microbial Biotechnology Researcher Center, Ethiopia.
[2]Ethiopian Agricultural Research Council Secretariat, Addis Ababa, Ethiopia
Corresponding author: betemariam21@gmail.com

ABSTRACT

In Ethiopia spontaneous fermentation is the sole means of making fermented food, which is prone to contamination and under quality product. Hence, the present study aimed at isolating and evaluating starter function of lactic acid bacteria for milk fermentation. Twenty samples were aseptically collected from Holetta agricultural research center dairy farm, Biftu berga farmer's union, Holland dairy farm, Genesis dairy farm and Adama residential households. A total of 350 isolates were obtained on MRS agar media and repeatedly cultured till uniform colonies were obtained. The purified isolates were then tested for acidifying efficacy. From 350 isolates twenty isolates were able to produce sufficient acid for milk fermentation. Then the 20 isolates were selected and tested for acid tolerance ability at 3.5, 3.8, 4 and 4.5 pH being incubated at 37°C of for 0, 2 and 4 hours. Seven (37%) isolates out of twenty showed both tolerance and growth at 4hours incubation at all pH values and further tested for temperature stress at 15, 25, 45 and 55° for the same incubation period. Of the seven isolates four (57%) were able to grow at 45 and 55° C for 4 and 2hours of incubation, while three of them (AD11, G16 and BB13) were found to be growing at 45 and 55°C, and 3.8 and 4 pH as optimum growth parameter. Based on screening techniques conducted AD11, G16 and BB13 could possibly be potential candidates for starter culture development for dairy fermentation.

Key words: Fermentation, lactic acid bacteria, milk, starter culture

INTRODUCTION

Starter cultures, vital part of almost all fermented food products, are defined as preparation of live microorganisms to initiate and speed up fermentation in a well-controlled and predictive manner (Durso and Hutkins, 2003). The application of starter culture has an immense role in enhancing preservation, improving nutritional values, modifying organoleptic properties and increasing economic value. Although traditional fermentation has long history in the making of fermented food products, the use of well characterized microorganisms as starter culture enables to produce fermented food with sustainable quality and safety (Guinee and Kilcawley, 2017).

The core of milk fermentation is the breakdown of lactose into lactic acid by microorganisms from previously fermented milk products, spontaneous proliferation of natural microbiota of milk or addition of well-defined starter cultures. The fundamental principle of lactic acid bacteria in fermentation attributes to their ability in degrading carbohydrates and produces lactic acid as a major end product. Besides, heterofermntative lactic acid bacteria are responsible for the formation of alcohols, aldehydes, acids, esters and sulphur compounds imparting specific organoleptic characteristic of fermented food products (Solange *et al.*, 2014; Widyastuti *et al.*, 2014; Bintsis, 2018).

Owing to the ability of LAB in hetero and homo fermentation of carbohydrates, they are applied as starter culture in fermentation of diverse array of dairy products viz. cheese, yoghurt, fermented milks and other food staffs including meat, fish, fruit, vegetable and cereal products providing the products with specific flavor, texture and nutritional value. In addition, the synthesis of exopolysaccharides during the course of fermentation are responsible for ripening of cheese, improving yoghurt texture and prevent secondary fermentation in winery. Besides, lactic acid bacteria are known in production of bacteriocins and antifungal compounds to enhance the shelf life of fermented products, and the health benefits in combating pathogenic microbes after consumption make them potential therapeutic probiotic cultures in food industry (Bintsis, 2018). The safe historical use of lactic acid bacteria is recognized by international organization, WHO, joint committee of FAO and WHO, European commission gave them the status of generally regarded as safe (GRAS) (Leroy and Vuyst, 2004).

Ethiopia has various traditional fermented milk products through the growth of indigenous wild microbiota of raw milk at ambient temperature. Fermentation using back slope techniques is well known as a major production method in Ethiopia. Nevertheless, application of well-defined and beneficial microorganisms to improve the quality of the product and enhance shelf life is seldom practiced in the country. Thus, the underestimated practices of the use of starter culture have been the cause of compromised quality and safety of dairy products and development of dairy industry in the country Daniel *et al*. (2019). Therefore, it is paramount important to develop potential starter culture for dairy fermentation to reduce post-harvest losses in the production of fermented dairy products along the dairy value chain and to improve the competitiveness of the dairy market in the country.

The ever-increasing population of the country boosts the need for high quality food products. In this regard, fermented dairy products play pivotal role in improving nutrition and protection against infectious diseases Tesfamariam *et al*. (2017). Therefore, it is indispensable to develop starter culture to enhance the production of dairy products with essential nutrients and public health important metabolites to alleviate malnutrition and related diseases.

The production of metabolites responsible for good quality of fermented food is directly related to rapid growth. In turn, the most important environmental parameters on which microbial growth depend are pH and temperature. The quality of milk products is affected by the response of lactic acid bacteria starter culture to production temperature and pH that determine rate of acidification and production of other metabolites responsible for organoleptic properties of fermented dairy products Ahmed *et al*. (2006). Moreover, the feedback inhibition of lactic acid hinders the synthesis of essential compounds for quality products. Therefore, the present study focused on determining optimum temperature and pH for lactic acid bacteria to determine starter culture potential for further application in dairy industry, small scale production and household usage.

MATERIALS AND METHODS

Description of Study area

The study was conducted at Holetta National Agricultural Biotechnology Research Center. Since dairy factories and unions are found in Bishoftu (8.7346° N, 39.0085° E), Adama (8.5263° N, 39.2583° E) and around Holetta (9”00 N and 38^{o} 30 E), samples were collected from these areas. Moreover, union and dairy factories are relatively closer to the laboratories to have fresh samples.

Milk sample collection and lactic acid bacteria isolation

Ten raw cow milk and ten ergo samples (1000ml) were aseptically collected with sterile bottle from areas of Addis Ababa milkshed and Adama viz., Holetta Research center dairy farm, Biftu berga dairy processing and manufacturing farmer’s union, Bishoftu (Ada’a dairy cooperatives, Holland dairy PLC, Genesis dairy processing farm) and Adamma (Household

composite sample) which were kept at +4°C refrigerator.

The raw milk and yogurt were serially diluted in saline solution up to 10^{-7}dalution. Selective media for lactic acid bacteria de Morgan Rogers's Sharp media (MRS) was prepared according to manufacturer's instruction and sterilized at 121°C, 15bar PSI for 15 minutes. One hundred microliter (100µl) of 10^{-4} up to 10^{-6} diluent was plated on MRS agar in triplicate and incubated overnight at 37°C. Multiple colonies were picked considering the colony characteristics (size, shape, color, texture etc) and grown in MRS broth overnight. Then subsequently streaked on MRS agar media until the colonies and cells were found to be uniform and pure Vázquez-Velázqueza *et al*. (2018).

Maintenance of the culture

The purified colonies of lactic acid bacteria were maintained in MRS broth according to the modified method described by Kumar *et al*. (2013). MRS broth was prepared based on predetermined instruction and dispensed in to test tubes from 5–10 ml and autoclaved at 121°C 15bar PSI for 15 minutes. After cooling, each test tube was heavily inoculated with pure fresh overnight culture of the isolates (4%, v/v) and incubated at 37°C for 24 hours, which were then stored in refrigerator (4°C). Prior to test procedures 2-3 transfers were made to fresh medium. Similarly, for long term preservation Non-Fat Skim Milk (NFM) was prepared and autoclaved at 121°C,15bar PSI for 5 minutes. Ten milliliter of non-fat skim milk was inoculated with fresh cultures of isolates and incubated at 37°C for overnight. The culture was harvested by centrifugation at 10,000xg for 5 minutes and the pellet was washed with phosphate buffer saline (PBS) solution. Then, the pellet was suspended into 10% NFM containing Glucose, Yeast extract and Glycerol (10% v/v) and finally stored in a freezer (-20°C).

Gram staining

A thin smear of each of the pure 24hourrs old culture was prepared on clean grease-free slides, fixed by gently passing over flame. Each heat-fixed smear was stained by addition of 2 drops of crystal violet solution for 60 sec and rinsed with water. The smeared glass slides were again flooded with lugol"s iodine for 30 sec and rinsed with water, decolorized with 70% alcohol for 15 sec and rinsed again with distilled water. They were then counter stained with 2 drops of safranin for 60 sec and finally rinsed with water, then allowed to air dry. The smears were mounted on a microscope and observed under oil immersion objective lens. Gram negative bacteria appeared pink or red, while gram positive bacteria appeared purple Fawole *et al*. (2004).

Catalase test

A loopful of 24hours old culture was transferred into a drop of 3% hydrogen peroxide solution on a clean glass slide with the aid of sterile inoculating loop. Gas bubbles seen as white froth indicates the presence of catalase enzyme (Cheesbrough, 2006).

Screening methods

Preliminary screening method

The major criterion in screening lactic acid bacteria for starter culture in dairy fermentation is capability of acidifying milk. The acidification study was done with a color change of bromocresol purple indicator, which changes color from purple to yellow in the presence of enough acid. MRS broth was prepared and supplemented with bromocresol purple (0.04gm/1000ml) for indicating acid production by inoculated microbes. Microtiter plates were filled with ninety micro liter (90µL) MRS broth enriched with bromocresol purple and inoculated with standardized one ten microliter (10 µL) (0.1-1 OD_{630}) fresh culture of the isolates. Culture with no bromocresol purple as a positive control and culture free bromocresol purple as negative control were employed to compare the color change. Isolates with color change were assumed to produce lactic acid and were allowed to pass to secondary screening Caˆmara1 *et al.* (2018).

Secondary screening

Acid stress test

A method employed by Ayo-Omogie and Okorie (2016) was used to study the acid tolerant efficacy of the isolates. Isolates with sufficient acid production were subjected to acid stress study. MRS broth was adjusted at pH 3.5, 3.8, 4.0 and 4.5 with HCl before sterilization at 121°C, 15bar PSI for 15 minutes. Nine hundred microliter (900 µL) of acid supplemented MRS broth was dispensed into deep well plates and inoculated with hundred microliters (100 µL) fresh (18 hours old) and standardized culture of isolates. MRS broth with optimum pH (6.0±0.02.) was inoculated with fresh culture as positive control and non-cell broth used as negative control. The whole set up was incubated at optimum temperature (37°C). A 100 µL from each well were taken at 0-, 2- and 4-hours interval, and read in microtiter reader at 630nm.

Temperature stress test

Isolates that survived acid stress were subjected to different levels of temperature stress: of 15, 25, 45 and 55°C, respectively. Nine hundred microliter freshly prepared MRS broth was filled into sterile deep-well plates, inoculated with hundred microliters (100 µL) of refreshed young (18 hour old) culture in 1:10 ration and incubated at 15, 25, 45 and 55°C. One hundred microliter (100 µL) was taken and read with microtiter plate reader at 630 nm (nanomer) and their respective growth were compared to control culture set at 37°C and pH 6.0±0.02 (Ayo-Omogie and Okorie, 2016).

Data analysis

One-way analysis of variance (ANOVA) was done using SAS 9.3 software. Significant difference between treatments mean was tested at P< 0.0 5 level of significance using Tukey's test.

RESULTS AND DISCUSSION

Lactic acid bacteria isolation and preliminary screening

Three hundred fifty (350) confirmed lactic acid bacteria (Fig 1A), based on gram staining (gram positive) and catalase production(catalase negative), were purified and tested for acid production (acidification).

Acidity is the most significant quality indicator for fermented milk as to the texture and flavor relay on optimum level of acidity. The acidity at the required level is also important in inhibiting spoilage bacteria and food borne pathogens (Huang *et al*., 2020). Hence, the present study focused on acidifying efficacy of lactic acid bacteria isolates. The color change of MRS broth supplemented with bromocresol purple from purple to yellow (Figure 1B) in which isolate under investigation grew is positive result for acid production. Based on which twenty among 350 isolates were found to produce acid and passed to the secondary screening acid stress and temperature stress study. The variation in acidifying ability among isolates attributes to the capability of degrading carbon and nitrogen sources in the media and processing the available nutrient Cheng *et al*. (2019). Besides, the existing nutrient transport system of the cells plays determinantal role in acidifying potential of lactic acid bacteria isolates as summarized by Fguiri *et al*. (2015).

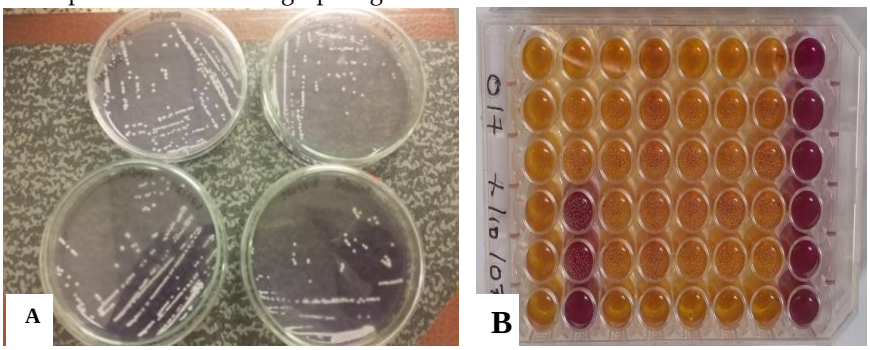


Figure 1. Colonies of lactic acid bacteria on MRS plates (A) and deep well plates with results of acidification test (B).

Acid stress test

The ability of tolerating specific acidic condition is an essential characteristic of isolates for starter culture candidacy of lactic acid bacteria. The lactic acid produced may have a feedback inhibition effect on the growth of candidate isolate and render the formation of crucial metabolites for organoleptic properties of the product Othman *et al*. (2017). Thus, acid tolerant ability of twenty lactic acid bacteria was evaluated.

The isolates have shown significant variation for each pH and time as depicted in table 1. Isolates BB13, DZ11, DZ10, AD11, DZ13, G16 and AD13 are the top 7 isolates with outstanding growth for 2 and 4 hours of incubation. Isolates capable of growing at lower pH for long period of time are supposed to be efficient isolate for starter culture. Hence, the mean absorbance value after 4hours of incubation at pH 4 was the highest for BB13 (0.8352 ±0.0887) and G16 (0.6870 ±0.0496), followed by DZ11 (0.8137 ± 0.1365) and DZ10 (0.7920 ±0.0367) for 2hours of incubation at the same pH condition. Whereas AD11 (0.7761± 0.0322) and AD13 (0.6601 ± 0.0251) were isolates with highest growth at pH 3.8 during 4hours of incubation. The survival and growth of isolates of the present is comparable to lactic acid bacteria reported by Cho *et al*. (2018) that reduced pH from 6.5 up to 3.8 and survived acidic conditions during milk fermentation.

The effect of pH is more pronounced in synthesis of major metabolites responsible for organoleptic properties of milk products. The rapid growth of lactic acid bacteria by lactose fermentation results in acidification (pH 5.5) which is responsible for dissociation of casein protein. The curdling of milk protein imparts pleasant texture of milk products. The more acidification the more curdling and good product texture Sinagaa *et al*. (2016). The survival and growth of lactic acid bacteria at pH 5.5 to 4.6 is vital for curdling process. Fortunately, the lactic acid bacteria in the present investigation can tolerate pH up to 4 (Figure 3) that makes them good starter culture for milk fermentation.

Table 1. Absorbance (OD) of LAB grown in MRS broth at different pH and incubation time

Isolate	Time (hr)	OD			
		pH=3.5	pH=3.8	pH=4	pH=4.5
AD1	0	0.5884 ± 0.0075^{b}	0.5777 ± 0.0204^{b}	0.5730 ± 0.0055^{b}	0.5176 ± 0.0048^{b}
	2	0.5723 ± 0.0766^{b}	0.5523 ± 0.0220^{b}	0.5719 ± 0.0040^{b}	0.5671 ± 0.0203^{b}
	4	0.5790 ± 0.0115^{b}	0.5639 ± 0.0223^{b}	0.5777 ± 0.0105^{b}	0.6531 ± 0.0386^{c}
AD11	0	0.5317 ± 0.0032^{b}	0.6932 ± 0.0403^{c}	0.5437 ± 0.0069^{b}	0.5353 ± 0.0432^{b}
	2	0.5473 ± 0.0269^{b}	0.6688 ± 0.0147^{c}	0.5614 ± 0.0093^{b}	0.5771 ± 0.0306^{b}
	4	0.5350 ± 0.0092^{b}	0.7761 ± 0.0322^{d}	0.5650 ± 0.034b	0.6045 ± 0.0262^{c}
AD13	0	0.5341 ± 0.0131^{b}	0.6325 ± 0.0124^{c}	0.5211 ± 0.0156^{a}	0.5156 ± 0.0094^{a}
	2	0.5547 ± 0.0060^{b}	0.5912 ± 0.0638^{bc}	0.5491 ± 0.0082^{b}	0.5788 ± 0.0443^{b}
	4	0.5260 ± 0.0246^{b}	0.6601 ± 0.0251^{c}	0.5167 ± 0.0274^{b}	0.6198 ± 0.0648^{c}
AD14	0	0.5881 ± 0.0071^{b}	0.5390 ± 0.0401^{b}	0.5653 ± 0.0050^{b}	0.7286 ± 0.3643^{d}
	2	0.5673 ± 0.0095^{b}	0.5440 ± 0.0348^{b}	0.5592 ± 0.0023^{b}	0.5705 ± 0.0271^{b}
	4	0.5240 ± 0.0026^{b}	0.5529 ± 0.0139^{b}	0.5583 ± 0.0006^{b}	0.5895 ± 0.0365^{b}
BB1	0	0.5316 ± 0.0049^{b}	0.5470 ± 0.0274^{b}	0.5614 ± 0.0096^{b}	0.5541 ± 0.0427^{b}
	2	0.5088 ± 0.0076^{ab}	0.5391 ± 0.0383^{b}	0.5629 ± 0.0140^{b}	0.5415 ± 0.0066^{b}
	4	0.5010 ± 0.0012^{ab}	0.5623 ± 0.0295^{b}	0.4891 ± 0.0197^{a}	0.5602 ± 0.0136^{b}
BB10	0	0.5039 ± 0.0116^{ab}	0.5240 ± 0.0138^{b}	0.5600 ± 0.0348^{b}	0.6468 ± 0.0208^{c}
	2	0.5114 ± 0.0032^{b}	0.5138 ± 0.0090^{b}	0.5949 ± 0.0622^{bc}	0.6502 ± 0.0278^{c}
	4	0.5336 ± 0.0131^{b}	0.5293 ± 0.0115^{b}	0.6341 ± 0.2668^{c}	0.6506 ± 0.0132^{c}
BB12	0	0.6303 ± 0.0483^{c}	0.5203 ± 0.0060^{b}	0.5373 ± 0.0172^{b}	0.5290 ± 0.0056^{b}
	2	0.5040 ± 0.0017^{b}	0.5067 ± 0.0006^{b}	0.5817 ± 0.0035$_{b}$	0.5050 ± 0.0010^{ab}
	4	0.5927 ± 0.0202^{bc}	0.5597 ± 0.0202^{b}	0.6097 ± 0.0637^{bc}	0.5067 ± 0.0006^{b}
BB13	0	0.5753 ± 0.0163^{b}	0.5676 ± 0.0176^{b}	0.6273 ± 0.0131^{b}	0.5993 ± 0.0471^{bc}
	2	0.5122 ± 0.0035^{b}	0.5306 ± 0.0179^{b}	0.6739 ± 0.0156^{c}	0.5878 ± 0.0429^{b}
	4	0.6628 ± 0.0457^{c}	0.5356 ± 0.0226^{b}	0.8352 ± 0.0887^{e}	0.5830 ± 0.0156^{b}
DZ1	0	0.5757 ± 0.0212^{b}	0.5667 ± 0.0499^{b}	0.5975 ± 0.0098^{bc}	0.5628 ± 0.0136^{b}
	2	0.6529 ± 0.0298^{c}	0.5892 ± 0.0398^{bc}	0.6217 ± 0.0111^{c}	0.5538 ± 0.0085^{b}
	4	0.5574 ± 0.0118^{b}	0.6432 ± 0.1382^{c}	0.6170 ± 0.0257^{c}	0.5927 ± 0.0384^{b}
DZ10	0	0.5243 ± 0.0025^{b}	0.5843 ± 0.0131^{b}	0.4978 ± 0.0006^{ba}	0.5614 ± 0.0772^{b}
	2	0.6440 ± 0.2272^{c}	0.7139 ± 0.1661^{cd}	0.7920 ± 0.0367^{d}	0.6254 ± 0.0824^{c}
	4	0.5193 ± 0.0149^{b}	0.6071 ± 0.0142^{bc}	0.5243 ± 0.0060^{b}	0.6368 ± 0.1015^{c}
DZ11	0	0.5183 ± 0.0075^{b}	0.5387 ± 0.0191^{b}	0.5031 ± 0.0051^{ab}	0.5617 ± 0.0688^{b}
	2	0.5227 ± 0.0065^{b}	0.5779 ± 0.0112^{b}	0.8137 ± 0.1365^{e}	0.6557 ± 0.0703^{c}
	4	0.5127 ± 0.0155^{b}	0.5694 ± 0.0103^{b}	0.5183 ± 0.0101^{b}	0.6354 ± 0.0558^{c}
DZ13	0	0.5223 ± 0.0032^{b}	0.6290 ± 0.0091^{c}	0.5028 ± 0.0081^{b}	0.5257 ± 0.0310^{b}
	2	0.5260 ± 0.0075^{b}	0.6133 ± 0.0123^{c}	0.5283 ± 0.0266^{b}	0.6897 ± 0.1561^{bc}
	4	0.5170 ± 0.0327^{b}	0.6101 ± 0.0056^{c}	0.5360 ± 0.0038^{b}	0.6511 ± 0.0890^{c}
G10	0	0.5033 ± 0.0031^{ab}	0.5089 ± 0.0017^{ab}	0.4857 ± 0.0081^{ab}	0.6569 ± 0.1868^{c}
	2	0.5005 ± 0.0075^{ab}	0.4976 ± 0.0035^{ab}	0.4676 ± 0.0068^{a}	0.5348 ± 0.0090^{b}
	4	0.5586 ± 0.0605^{b}	0.5058 ± 0.0060^{ab}	0.5401 ± 0.0207^{b}	0.5503 ± 0.0137^{b}
G11	0	0.6807 ± 0.0251^{c}	0.6468 ± 0.0180^{c}	0.5953 ± 0.0887^{bc}	0.5688 ± 0.0006^{b}
	2	0.6351 ± 0.0506^{c}	0.6343 ± 0.0199^{c}	0.5948 ± 0.0243^{bc}	0.5173 ± 0.0345^{b}
	4	0.6838 ± 0.0188^{c}	0.5209 ± 0.0068^{b}	0.6028 ± 0.0159^{bc}	0.5195 ± 0.0182^{b}
G13	0	0.5600 ± 0.0030^{b}	0.5527 ± 0.0182^{b}	0.5345 ± 0.0185^{b}	0.5374 ± 0.0060^{b}
	2	0.5609 ± 0.0416^{b}	0.5285 ± 0.0139^{b}	0.5611 ± 0.00 36^{b}	0.5301 ± 0.0564^{b}
	4	0.5924 ± 0.0127^{b}	0.5959 ± 0.0117^{b}	0.5716 ± 0.0207^{b}	0.5480 ± 0.0127^{b}
G16	0	0.6273 ± 0.0241^{c}	0.5820 ± 0.0026^{b}	0.6262 ± 0.0826^{c}	0.5918 ± 0.0142^{b}
	2	0.5412 ± 0.0306^{b}	0.5462 ± 0.0133^{b}	0.6530 ± 0.0581^{c}	0.5975 ± 0.0118^{b}
	4	0.6648 ± 0.0186^{c}	0.6529 ± 0.0281^{c}	0.6870 ± 0.0496^{c}	0.5987 ± 0.0228^{b}
NZ1	0	0.5750 ± 0.0075^{b}	0.562 ± 0.0082^{b}	0.5395 ± 0.0225^{b}	0.5518 ± 0.0031^{b}
	2	0.5736 ± 0.0427^{b}	0.5345 ± 0.0340^{b}	0.5627 ± 0.0080^{b}	0.5595 ± 0.0105^{b}
	4	0.5491 ± 0.0157^{b}	0.6212 ± 0.0321^{b}	0.5623 ± 0.0055^{b}	0.5444 ± 0.0095^{b}
NZ10	0	0.5066 ± 0.0026^{ab}	0.4996 ± 0.0023^{ab}	0.5033 ± 0.0080^{ab}	0.5269 ± 0.0055^{b}
	2	0.5008 ± 0.0010^{ab}	0.5300 ± 0.0070^{b}	0.4459 ± 0.0031^{ab}	0.5132 ± 0.0038^{ab}
	4	0.5173 ± 0.0079^{ab}	0.5632 ± 0.0243^{b}	0.5621 ± 0.0125^{b}	0.5023 ± 0.0046^{ab}
NZ11	0	0.5980 ± 0.0163^{bc}	0.5160 ± 0.0114^{ab}	0.5985 ± 0.0029^{bc}	0.5621 ± 0.0347^{b}
	2	0.5757 ± 0.0395^{bc}	0.5320 ± 0.0171^{b}	0.6325 ± 0.0249^{c}	0.6217 ± 0.0428^{c}
	4	0.6037 ± 0.0329^{c}	0.4980 ± 0.0012^{ab}	0.6224 ± 0.0200^{b}	0.6091 ± 0.0597^{bc}
NZ12	0	0.5039 ± 0.0049^{ab}	0.5096 ± 0.0076^{ab}	0.4733 ± 0.0026^{a}	0.5583 ± 0.0203^{b}
	2	0.5032 ± 0.0029^{ab}	0.5480 ± 0.0235^{b}	0.4436 ± 0.0006^{a}	0.5242 ± 0.0075^{b}
	4	0.5376 ± 0.0586^{b}	0.5402 ± 0.0293^{b}	0.5901 ± 0.0334^{bc}	0.5137 ± 0.0090^{b}

Abbreviation indicating sampling areas from which isolates were obtained AD (Ada'a), BB (Biftu berga), DZ (Debrezeit), G (Genesis) and NZ (Nazriet)

A report by Guan *et al.* (2016) more efficient lactic acid bacteria growth at pH 2.5 after 4hours of incubation at 37°C, whereas the highest growth in current report occurred at pH4 by BB13 (0.8358±0.0887). The result of Nawaz *et al*. (2017) aligned with the present study where the survival and growth of most of the isolates at pH 3 and 4 was remarkable. The acidification capability of LAB isolates of up to pH 5 -5.5 was reported as the best pH for milk coagulation Lucey (2016). This implies that the capability of growth and tolerance of the isolates in the present study, pH of 4 (Figure 2) and temperature of 37°C make them potential candidates for the development of dairy starter culture.

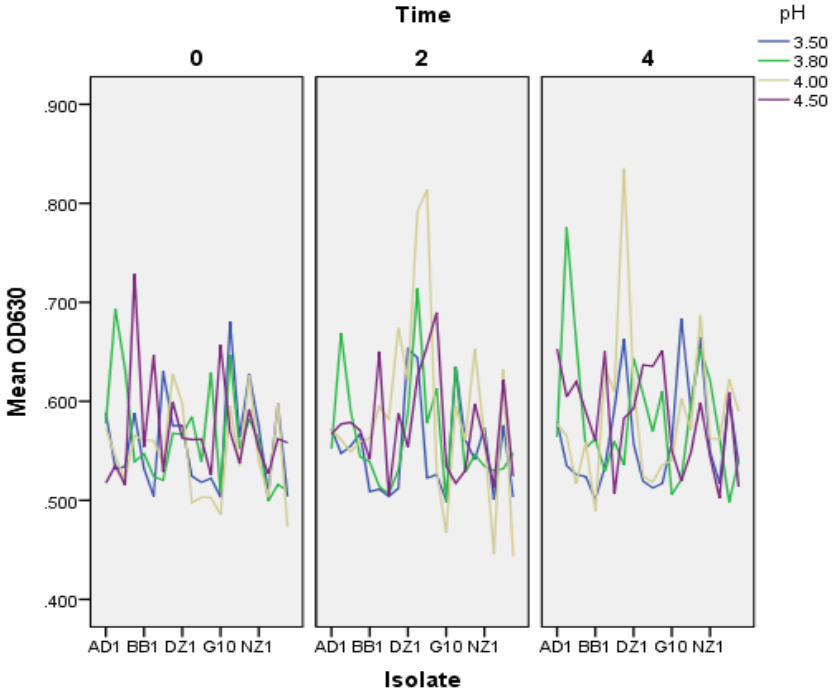


Figure 2. Growth pH preference of lactic acid bacteria while growing at 3.5, 3.8, 4 and 4.5 pH at 37°C for 0, 2 and 4 hours. .

Temperature stress test

Like acid stress test, the isolates with high absorbance for extended incubation period are the best candidates for starter culture development (Table 2). The isolates have shown significant variation at each temperature and incubation period. The survival and growth of isolates at 45°C and 55°C, and for 4 hours of incubation period were the highest compared to those isolates incubated at 15 and 25 °C. Based on temperature study, 57% of the isolates viz., AD11 (0.7107 ± 0.0204), G16 (0.6103 ± 0.1022), BB13 (0.5663 ±0.0086) and DZ10 (0.5703 ± 0.1019) are the potential candidates for milk fermentation at higher temperatures of 45 and 55 °C (Figure 3). The isolates could possibly be employed in low land areas of temperature above 40°C and suitable for industrial scale milk fermentation as well. The result of this study coincide with the findings of Ibrahim *et al*., (2019) that the optimum temperature for cow and goat milk fermentation is 46 °C. The impact of fermentation temperature on product quality was studied by Ostlie *et al*. (2004) which revealed the variation on fermentation temperature brought about significant influence on the metabolite products responsible for organoleptic properties of milk products after 48hours of incubation at 30, 37 and 45°C. The highest metabolites were produced at 45°C for 48hours of incubation. The researchers recommended the importance of controlling fermentation temperature at optimum level that supports the aim of the present study.

Furthermore, G16 (0.5200 ± 0.0044) and BB13 (0.5107 ± 0.0006) were able to grow at incubation temperature of 15°C for 4 and 2 hours respectively. The result implied that the LAB isolates can be used as starter culture for small scale and household milk fermentation, where heating incubation facilities are lacking. Moreover, these isolates could be used in highland areas of the country with low temperature range.

Table 2: Absorbance (OD_{630}) of LAB grown in MRS broth at varying temperature and incubation period

Isolates	Time in hour	OD			
		15°C	25°C	45°C	55°C
AD11	0	0.5130 ± 0.0017^{ab}	0.4990 ± 0.0036^{ab}	0.5027 ± 0.0042^{ab}	0.5063 ± 0.0042^{ab}
	2	0.5023 ± 0.0012^{ab}	0.4970 ± 0.0036^{ab}	0.5027 ± 0.0031^{ab}	0.5090 ± 0.0017^{ab}
	4	0.5027 ± 0.0081^{ab}	0.5197 ± 0.0031^{ab}	0.7107 ± 0.0204^{d}	0.5137 ± 0.0093^{ab}
AD13	0	0.5037 ± 0.0021^{ab}	0.5067 ± 0.0038^{ab}	0.7107 ± 0.0112^{d}	0.5023 ± 0.0021^{ab}
	2	0.5107 ± 0.0006^{ab}	0.5147 ± 0.0081^{ab}	0.5247 ± 0.0015^{b}	0.5123 ± 0.0029^{ab}
	4	0.5067 ± 0.0021^{ab}	0.5340 ± 0.0181^{b}	0.5383 ± 0.0040^{b}	0.5223 ± 0.0015^{b}
BB13	0	0.5203 ± 0.0025^{b}	0.4727 ± 0.0055^{a}	0.5200 ± 0.0010^{b}	0.5327 ± 0.0311^{b}
	2	0.5180 ± 0.0044^{ab}	0.5070 ± 0.0026^{ab}	0.5307 ± 0.0015^{b}	0.5287 ± 0.0199^{b}
	4	0.5117 ± 0.0112^{ab}	0.4590 ± 0.0010^{a}	0.5383 ± 0.0021^{b}	0.5663 ± 0.0086^{b}
DZ10	0	0.5430 ± 0.0141^{b}	0.5337 ± 0.0064^{b}	0.5377 ± 0.0253^{b}	0.5717 ± 0.0129^{b}
	2	0.5050 ± 0.0012^{ab}	0.5317 ± 0.0155^{b}	0.5703 ± 0.1019^{b}	0.5127 ± 0.0045^{ab}
	4	0.4930 ± 0.0010^{ab}	0.5163 ± 0.0006^{ab}	0.5703 ± 0.0050^{b}	0.5007 ± 0.0038^{ab}
DZ11	0	0.5227 ± 0.0197^{b}	0.5253 ± 0.0040^{b}	0.5300 ± 0.0272^{b}	0.6107 ± 0.0146^{c}
	2	0.5050 ± 0.0017^{ab}	0.5163 ± 0.0121^{ab}	0.5083 ± 0.0040^{ab}	0.5127 ± 0.0044^{ab}
	4	0.4947 ± 0.0025^{ab}	0.5143 ± 0.0040^{ab}	0.5600 ± 0.0182^{b}	0.5030 ± 0.0035^{ab}
DZ13	0	0.5637 ± 0.0096^{b}	0.5423 ± 0.0131^{b}	0.5227 ± 0.0015^{b}	0.6057 ± 0.0293^{c}
	2	0.5093 ± 0.0040^{ab}	0.5170 ± 0.0140^{ab}	0.5120 ± 0.0036^{ab}	0.4987 ± 0.0029^{ab}
	4	0.5103 ± 0.0292^{ab}	0.5303 ± 0.0121^{b}	0.5030 ± 0.0383^{ab}	0.4947 ± 0.0012^{ab}
G16	0	0.5093 ± 0.0072^{ab}	0.5050 ± 0.0010^{ab}	0.5030 ± 0.0079^{ab}	0.5083 ± 0.0084^{ab}
	2	0.5177 ± 0.0055^{ab}	0.5140 ± 0.0078^{ab}	0.5370 ± 0.0046^{b}	0.6103 ± 0.1022^{c}
	4	0.5200 ± 0.0044^{b}	0.5123 ± 0.0006^{ab}	0.5427 ± 0.0031^{b}	0.5533 ± 0.0049^{b}

Abbreviation stand for isolate names from sample of origin: AD(Ada'a), BB(Biftu Berga), DZ(Debre Zeit)

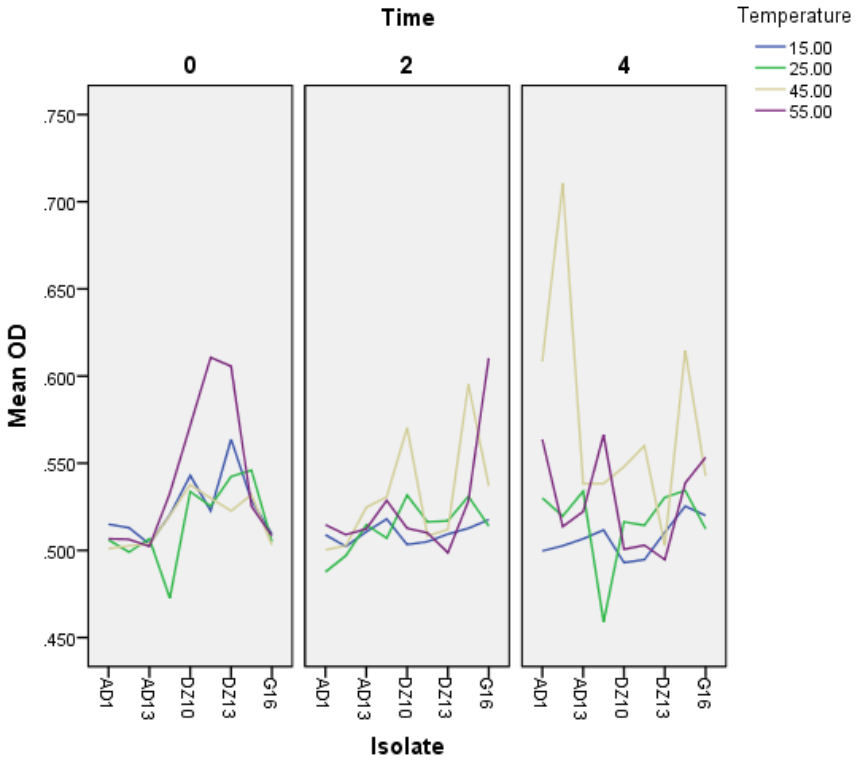


Figure 3. Growth temperature preference of lactic acid bacteria while growing at 15, 25, 450 and 55°C for 0, 2 and 4hours.

Generally, AD11 is the outstanding isolate in tolerating and growing at 3.8 pH and 45°C followed by G16 which withstood and grew at pH of 4 for 4hours and incubation temperature of 55°C for 2hours. In addition, BB13 grew at pH of 4 and during 4hours of incubation at 55°C. Although most isolates were capable of growing at 45 and 55°C and at pH of 4, three (AD11, G16 and BB13) were found to be the best isolates in both acid tolerance and temperature stress. Hence, temperature 45 and 55°C, and 3.8 and 4 pH are found to be optimum growth conditions for milk fermentation by AD11, G16 and BB13 isolates.

CONCLUSION

The production and quality of fermented dairy products relies on utilization of efficient starter culture. In the present study isolation of thermophilic lactic acid bacteria from traditional raw and traditional fermented milk products particularly 'ergo' was achieved. The best growth of lactic acid bacteria isolates was observed at 45 and 55°C at 3.8 and 4pH with excellent acidification. Three isolates AD11, G16 and BB13 were found to be a potential starter culture candidates based on their temperature and tolerance of pH stress.

ACKNOWLEDGMENT

The authors would like to acknowledge Ethiopian Institute of Agricultural Research for funding the project and providing with laboratory facilities.

REFERENCES

Ahmed, T, Kanwal, R and Ayub, N. 2006. Influence of temperature on growth pattern of Lactococcus lactis, Streptococcus cremoris and Lactobacillus acidophilus isolated from camel milk. *Biotechnology* **5(4)**: 481-488.

Ayo-Omogie, HN and Okorie, EI. 2016. In vitro Probiotic Potential of Autochthonous Lactic Acid Bacteria and Microbiology of Kunu Made from Mixed *Grains. British Microbiology Research Journal.* **14(4):** 1-10.

Bintsis, T. 2018. Lactic acid bacteria: their applications in foods. *J Bacteriol Mycol Open Access.* **6(2):**89–9.

Caˆmara, SC, Dapkevicius, A, Riquelme, C, Elias, RB, Silva, CCG, FX Malcata, FX and MLNE Dapkevicius, MLNE. 2018. Potential of lactic acid bacteria from Pico cheese for starter culture development. *Food Science and Technology International.* **0(0)**: 1–15.

Cheesbrough, M. 2006. *District Laboratory practice in tropical countries*. 2nd ed., Cambridge University Press, Cambridge, UK. Othman, M, Ariff, AB, Rios-Solis, L and Halim M. 2017. Extractive Fermentation of Lactic Acid in Lactic Acid Bacteria Cultivation: A Review. *Front. Microbiol*. 8: 1-7.

Cheng, F, Chen, H, Lei, N, Zhang, M, Wan, H. 2019. Effect of carbon and nitrogen sources on activity of cell envelope on proteinase produced by *Lactobacillus plantarum* LP69. *Food technology*. **23**: 11-18.

Cho, G, Cappello, C, Schrader, K, Fagbemigun, O, Oguntoyinbo, AF, Csovcsics, C, Rösch, N, Kabisch, J, Neve, H, Bockelmann, W, Briviba, K, Modesto, M, Cilli, E, Mattarelli, P and Franz, CMAAP. 2018. Isolation and Characterization of Lactic Acid Bacteria from Fermented Goat Milk in Tajikista. *J. Microbiol. Biotechnol*. **28(11):** 1834-1845.

Daniel, G, Tewodros, A, Dawit, A, Mebrate, G and Derbie, Z. 2019. Probiotic bacteria in relation to Ethiopian dairy industry. *Microbiology Research International*. **7(3)**: 24-30.

Durso, L and Hutkins, R. 2003. Starter cultures. pp. 5583-5593 Elsevier Science Ltd.

Fawole, MO and Oso, BA. 2004. *Characterization of Bacteria: Laboratory manual of microbiology*. 4 th Edn., Spectrum Book Ltd., Ibadan, Nigeria.

Fguiri, I, Ziadi, M, Atigui, M, Ayeb, N, Arroum, S, Assadi, M and Khorchani, T. 2015. Isolation and characterization of lactic acid bacteria strains from raw camel milk for potential use in the production of fermented Tunisian dairy products. *International Journal of Dairy Technology.* **68**: 1-11.

Guan, X, Xu, Q, Zheng, Y, Qian, L and Lin, B. 2017. Screening and characterization of lactic acid bacterial strains that produced fermented milk and reduce cholesterol. *Brazilian Journal of Microbiology* **48**: 730-739.

Guinee, PT and Kilcawley, NK. 2017. Ingredient Cheese and Cheese-Based Ingredients. pp. 715-755. *In* Cheese: Chemistry, Physics and Microbiology Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland.

Ibrahim, IA, Naufalin, R, Erminawati and Dwiyanti, H. 2019. Effect of fermentation temperature and culture concentration on microbial and physicochemical properties of caw and goat milk yogurt. *IOP Conf. Series: Earth and environmental science* **406**: 1-7.

Kumar, S, Kashyap, PL, Singh, R and Srivastava, AK. 2013. Preservation and Maintenance of Microbial Cultures. pp. 135-152. *In* Arora (eds.) Analyzing Microbes, Springer-Verlag Berlin Heidelberg.

Leroy, F and Vuyst, L. 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science and Technology* **15**: 67-68.

Lucey, JA. 2016. Acid coagulation of milk. pp.309-328. *In* O'Mahony, JA. and McSweeney, PLH. (eds.) Advanced Dairy Chemistry. Springer Science Business Media, New York.

Nawaz, ASN, Jagadeesh, K.S. and Krishnaraj, P.U. 2017. Isolation and screening of lactic acid bacteria for acid pH and bile tolerance. *International Journal of Current Microbiology and Applied Sciences* **7**: 3975 – 3980.

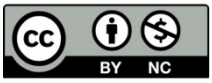
Østlie, HM, Treimo, J and Narvhus, JA. 2004. Effect of temperature on growth andmetabolism of probiotic bacteria in milk. *International Dairy Journal* **15**: 989–997.

Solange, AKA, Georgette, K, Gilbert, F, Marcellin, DJE K and Bassirou, B. 2014. Review on African traditional cereal beverages. *American Journal of Research Communication* **2(5)**: 103-153.

Tesfemariam, B, Kvist, FV, Ipsen, R, Eyassu, S, Mohammed YK and Bech, EH. 2017. Traditional Fermented Dairy Products of Ethiopia: A Review. *East African Journal of Sciences*. **11 (2):** 73-80.

Vázquez-Velázquez, R, Salvador-Figueroa, M, AdrianoAnaya, L, DeGyves–Córdova, G and Vázquez-Ovando, A. 2018. Use of starter culture of native lactic acid bacteria for producing an artisanal Mexican cheese safe and sensory acceptable. *Journal of Food*, **16**: 460-468.

Widyastuti, Y, Rohmatussolihat, and Febrisiantosa. 2014. The Role of Lactic Acid Bacteria in Milk Fermentation. *Food and Nutrition Sciences*. **5**: 435-442.



ISSN 2220-9325 (Online), ISSN 2203-5802(Print)