In Vitro Evaluation of the Probiotic Potential of Lactic Acid Bacterial Strains Retrieved from Raw and Traditionally Fermented Cow Milk

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Abstract

Background: Probiotics are live bacteria found mostly in milk and milk products that have been shown to improve intestinal microflora composition, treat lactose intolerance, prevent cancer, allergies, hepatic illness, and lower cholesterol. Ethiopians consume a lot of dairy and dairy products. However, little is known about the starter and probiotic properties of the lactic acid bacteria consumed with these items in the country.

Objective: The objective of this research was to identify and evaluate the probiotic functioning of lactic acid bacteria from raw and traditional fermented cow milk.

Materials and Methods: Lactic acid bacteria were isolated from raw milk and yoghurt samples collected from Ethiopia (Holetta, Adama and Bishoftu). Three hundred and fifty colonies exhibiting the characteristic features of lactic acid bacteria were used for gastric and bile salt tolerance tests.

Results: From among the 27 isolates, 10 (37%) showed a significant tolerance to the various ranges of gastric pH and bile salt concentrations ($P \leq 0.05$). The highest gastric acid tolerance was observed for the isolate AD6 (OD = 1.352 ± 0.063) at the gastric pH of 4.0 at 24th hours of incubation followed for the isolate NZ26 (OD = 0.870 ± 0.058) at the same gastric pH and incubation hour. Isolate G25 (OD = 0.733 ± 0.103) was able to tolerate 2% (w/v) of bile salt at 2 h of incubation time. Four isolates DZ3 (OD = 0.578±0.103), G37 (OD = 0.657 ± 0.046), AD22 (OD = 0.683 ± 0.072) and NZ3 (OD = 0.694 ± 0.070) showed a significance tolerance at 1% (w/v) of bile salt concentration at the 24th hours of incubation.

Conclusion: The findings revealed that naturally occurring lactic acid bacteria isolated from dairy products have the potential for probiotic applications in the dairy industry in the country.This could pave the way for exploiting the isolates at industrial level and could transform traditional dairy processing with probiotic function in Ethiopia.

Keywords: Bile salt; Dairy products; Gastric acid; Lactic acid bacterial; Probiotic potential

1. Introduction

Probiotics, according to the definitions given by Ejtahed *et al.* (2011) are live microorganisms that provide health benefits on the host. They are known to improve the composition of intestinal micro flora, relieve lactose intolerance, prevent cancer, allergies, hepatic disease and facilitate cholesterol (Yusuf *et al.,* 2018). Lactic acid bacteria are found in various traditional fermented foods such as dairy products. Lactic acid bacteria are currently the subject of extensive research due to their involvement in most traditional fermented foods and their potential to produce antimicrobial metabolites that enhance the shelf life of food products (Yeshambel *et al.,* 2021). In addition, the consumption of probiotics has been associated with enhanced immune response, reduced onset of enter pathogenic bacteria in the gut and diarrhoea (Reid, 1999). Previously, scientific investigations have supported a role for probiotics as a part of a healthy diet for humans and animals and may be an avenue to provide a safe and cost effective barrier

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ISSN 1993-8195 (Online), ISSN 1992-0407(Print)

against microbial infections (Parvez *et al.,* 2006). Dairy and food industries use metabolites of probiotic lactic acid bacterial for natural preservatives and flavour enhancers (Reid, 1999).

Lactic acid bacteria gained the reputation for being the main probiotic microbes. These beneficial microbes belong to a diverse bacterial group consisting of 11 genera. They are Gram-positive, non-spore-forming cocci or rods able to produce lactic acid as a by-product. Historically, lactic acid bacteria are considered GRAS (generally regarded as safe) microbes and especially members of the genus *Lactobacillus*, *Lactococcus* and *Streptococcus* are widely used in the food industry. Nowadays, various species of *Lactobacillus* have been used in food products as probiotic functioning organisms. Probiotic strains are selected for potential application on the basis of particular physiological and functional properties (Sanders *et al*., 1999). Probiotics are living, health-promoting microorganisms that are incorporated into various kinds of foods and the probiotic bacterial strains are generally provided with food system and then consumed orally, their passages set up from the mouth to the lower intestinal lumen, and thus the strains are required to overcome different stress conditions such as low-pH and bile in the gastrointestinal tract for survival and the beneficial effect (Hoque *et al*., 2010).

According to Sivapalasingam *et al.* (2004) the problem of food-borne diseases is multifactorial, and their prevention and control require multidisciplinary approaches that involve human beneficial live microbes (probiotics) in order to combat these pathogens and their associated health risks. Several in vitro studies indicated that probiotic lactic acid bacteria (Tesfaye *et al.,* 2011) inhibit the growth of food-borne pathogenic microbes. The consumption of a large number of probiotic live microorganisms together with a food fundamentally promotes the health of the consumers.

In Ethiopia, a considerable portion of milk is consumed in a fermented state as "Ergo". The fermentation is takes place naturally, without the use of defined starter culture to initiate the fermentation process and this is made only through the proliferation of normal microbial flora in the milk. In addition, little is known regarding the starter and the probiotic functions the microbes used in this regard. It does not have any definite temperature and duration of incubation. The development of microorganisms during ergo fermentation showed variations in various parameters. Despite limitations with dose and viability of probiotic strains, a lack of industry standardization, and potential safety concerns, according to Parvez *et al.,* 2006, there is clearly substantial promise for the benefits of probiotics across a wide range of clinical disorders. Basic research will continue to identify and characterize existing probiotic strains, as well as identify strain-specific outcomes, define the best dose for specific outcomes, and test their stability during processing and digestion.

Many people worked on isolating and screening antibacterial-producing lactic acid bacteria from traditionally fermented foods (Akalu *et al.,* 2017). Tesfaye *et al.,* 2011 discovered that lactic acid bacterial strains, either as pure or defined mixed cultures, exhibit antagonistic effects against some food-borne pathogens during the fermentation and storage of fermented milk. However, there is currently a scarcity of studies on probiotic lactic acid bacteria characterisation. The majority of Ethiopia's traditionally fermented items are ingested without further heat processing, making them suitable vehicles for transporting probiotic bacteria into the human gastrointestinal tract.Despite the fact that there has been a lot of study done on probiotics, there is still a need to find new strains because probiotic qualities are strain-specific.

Given the benefits of probiotics on child growth, using readily available and less expensive fermented food products as a vehicle for probiotics could play a significant role in improving nutrition, treating enteric infections, and promoting compensatory growth in children in developing countries via these various mechanisms. Before promoting fermented foods in supplemental feeding in underdeveloped nations, more research is needed regarding consumer confidence, acceptance of fermented goods as a source of probiotics, and safety issues (Sleator, 2010).The research hypothesis is that lactic acid bacteria with high probiotic activities can be found in dairy products such as raw milk and yogurt.Generally, dairy products are considered primary food sources for lactic acid bacteria probiotics. Fermented cow milks are consumed in different regions of the world. The presence of high counts of lactic acid bacteria in dairy products as beneficial micro biota indicates a source for explorations of biological materials of considerable health importance and vast applications in the dairy industry. Although researchers from other countries have screened and characterized lactic acid bacteria probiotic strains from various dairy products and food hence, in the present study, we aimed at isolating, characterizing and evaluating the probiotic functioning potential of LAB from indigenous Ethiopian dairy products.

2. Material and Methods

2.1. Chemicals and Media

Chemicals and reagents used in this study were de Man, Rogosa and Sharpe (MRS, Oxoid Ltd., Basingstoke, England) agar for *Lactobacillu*s and M17 broth and agar powder for *Lactococcus* isolation (HI Media, Mumbai, India). All experiments were conducted at the Holetta National Agricultural Biotechnology Research Centre, National Microbial Biotechnology Research Laboratory, Ethiopia.

2.2. Study design

Lactating dairy cattle were the study animals that were managed in a semi-intensive way. Isolation and characterization of lactic acid bacteria was done from raw milk and yoghurt obtained from lactating dairy cows in Holetta, Adama and Bishoftu towns in Central Ethiopia.

2.3. Sample Collection and Isolation of Lactic Acid Bacterial Strains

A total of twenty (20) milk samples (1000 ml) were collected from lactataing dairy cows from Holetta, Adama and Bishoftu) towns using sterile bottles. The milk samples were kept at 4°C before isolation. The samples were transported to the National Agricultural Biotechnology Research Centre, Holetta National Microbial Biotechnology Research Laboratory.

After 3–5 days of complete fermentation, the raw milk samples were serially diluted (1:10) using sterile saline [0.85% NaCl (w/v)]. The fermented samples were ready for serial dilution with no further fermentation needed. Hundred microliters (100µl) sampled from the serial dilutions (10^{-4}–10^{-7}) were spread on to de Man, Rogosa and Sharpe (MRS) and M17 agar media using glass road. The plates were incubated at 37 °C for 24–72 h anaerobically. Finally, colonies that exhibited the characteristics of lactic acid morphology (rod shaped cell, on sporulating, small and white colonies) were picked, and maintained as MRS and M17 broth for further study (Hoque *et al.*, 2010).

2.4. Preservation of Cultures of Pure Lactic Acid Bacteria Isolates

Tubes containing 5–10 ml of MRS or M17 broth were inoculated heavily with pure, fresh overnight cultures of the isolates (4% v/v) stored at 4–6 °C in a refrigerator) for short term preservation. For long period maintenance of isolates, 10% of skim milk powder was prepared and autoclaved at 121 °C for 5 minutes. Fresh lactic acid bacterial cultures from broth were inoculated into Eppendorf tubes containing 1 to 2 ml of skim milk. The tube was incubated at 37°C for 18 to 24 h. Cells from MRS and M17 broth were separated by centrifuge at 10000 rpm for 10 minutes. Then, the cell-free the supernatant was discarded and the pellets were suspended in 10% glycerol, then the tube was kept at –20 °C for further use.

2.5. Preliminary Screening of Lactic Acid Bacteria

Mass selection of LAB using deep well micro titration plates

Bacterial isolates were refreshed in MRS broth at 37 °C for 18 h and washed three times at 6000/5000 rpm for 10 minutes using normal saline solution (0.85% NaCl) to get rid of the broth media traces. The turbidity of bacterial suspension was adjusted to optical density (OD) of 0.1 to 0.5 using a spectrophotometer at 630 nm. Ninety-six well microtiter plates were filled with 990 µl of MRS broth supplemented with bromocresol purple (0.04 g/1000 ml) and were inoculated with 10 µl of the standardized LAB cultures. Culture-free wells served as a negative control. Finally, the plates were incubated at 37 °C for 18 to 24 h and absorbance was read at 630 nm and the formation of a yellow colour (indicating a positive result for fermentation or acid reduction efficiency of the strains) was examined visually.

2.6. Gastric Acid Tolerance Test

The ninety-six deep well microtiter plate method was used for evaluating the stomach gastric acid tolerance efficacy of the isolates according the method mentioned in (Suree *et al.*, 2012) de Man, Rogosa and Sharpe broth was used. The selected isolates were incubated in microtiter plates containing different pH values (2, 3 and 4) and samples were taken at 0, 2nd, 4th and 24th hour of incubation. The optical density (OD) of the broth was read at 630 nm and the results of the reading recorded.

2.7. Bile Salt Tolerance Test

The isolates were grown in de Man, Rogosa and Sharpe broth supplemented with 0.3%, 1%, 1.5% and 2% bile Oxgall with the pH adjusted to 7 and 8 (John and Alicia, 2011; Liong and Shah, 2005). The optical density (OD) of the incubated samples were read at 630 nm prior to 0 h, 2 h, 4 h and 24h of incubation against blank MRS with and without bile Oxgall (Gilliland and Walker,1990; Liong, 2006).

2.8. Data Analysis

The data were analysed using SAS statistical software packaged version 9.2 for windows. Results were presented as mean ± SD and one-way ANOVA was performed followed by turkey's post hoc test to separate means at 5% level of significance.

3. Results

3.1. Isolation of Lactic Acid Bacteria

A total of 350 lactic acid bacterial isolates were recovered from twenty different raw (10 raw) and fermented milk (10 yoghurt) of which 27 best performing isolates were selected for the probiotic functioning test.

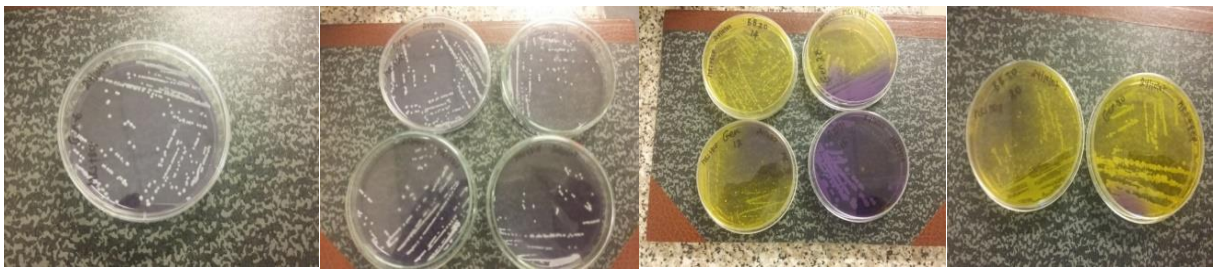

Figure 1. Colonies of lactic acid bacteria isolates on MRS (de Man, Rogosa and Sharpe Agar Media agar) plates.

3.2. Mass Screening of Lactic Acid Bacteria

The mass screening of all isolated lactic acid bacteria was done using MRS broth and Bromocresol purple as an indicator. The formation of a yellow colour indicated a positive result for fermentation or acidification whereas the absence of any colour change is considered as a negative result (Figure 2).

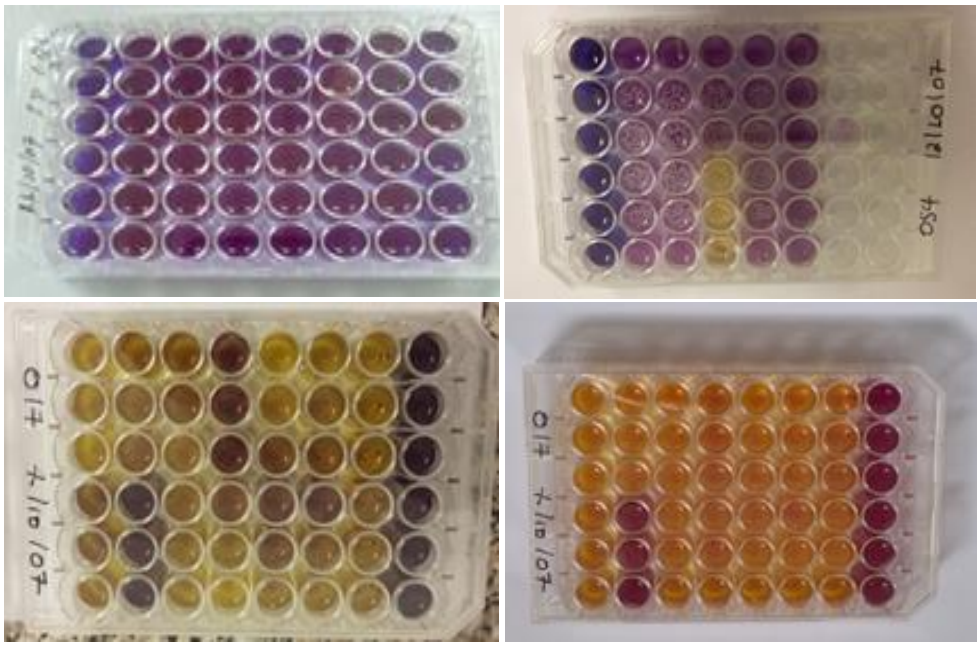

Figure 2. Mass selection of Lactic acid bacterial isolates using micro titration plates (MRS broth + BCP indicator).

3.3. *In Vitro* Analysis of Probiotics Properties of LAB

3.3.1. Gastric acid tolerance test

Among the 27 isolates 10 (37%) showed a significant tolerance to various ranges of gastric pH (2, 3 and 4, $P < 0.05$). Most of the isolates were able to tolerate various gastric pH and the highest gastric acid tolerance were observed for isolate AD6 (OD = 1.352 ± 0.063) at a gastric pH of 4 h at 24 h of incubation followed by NZ26 (OD = 0.870 ± 0.058) at the same gastric pH and incubation hour. The mean results are indicated here below at an absorbance of 630 nm (Table 1).

Table 1. Gastric acid tolerance test results of probiotic lactic acid bacteria.

Codes of isolates	Time of what (h)	Gastric pH		
		2	3	4
		Mean OD at 630 nm		
AD6	0	0.510±0.005	0.422±0.001	0.517±0.001
	2	0.495±0.006	0.596±0.115	0.523±0.006
	4	0.509±0.001	0.502±0.002	0.528±0.028
	24	0.667±0.108	0.510±0.003	1.352±0.063
NZ26	0	0.511±0.007	0.492±0.010	0.514±0.003
	2	0.489±0.001	0.498±0.003	0.510±0.009
	4	0.505±0.002	0.502±0.001	0.533±0.020
	24	0.726±0.094	0.501±0.002	0.870±0.058
BB26	0	0.507±0.003	0.725±0.034	0.511±0.006
	2	0.506±0.003	0.588±0.044	0.511±0.005
	4	0.512±0.004	0.502±0.005	0.510±0.001
	24	0.511±0.006	0.741±0.057	0.519±0.007
DZ9	0	0.519±0.007	0.503±0.008	0.546±0.027
	2	0.515±0.017	0.521±0.019	0.521±0.019
	4	0.504±0.010	0.502±0.001	0.524±0.012
	24	0.522±0.012	0.512±0.005	0.804±0.112
G4	0	0.520±0.018	0.491±0.004	0.546±0.053
	2	0.508±0.012	0.671±0.088	0.504±0.002
	4	0.506±0.005	0.501±0.001	0.512±0.008
	24	0.640±0.065	0.501±0.002	0.507±0.008
DZ5	0	0.508±0.001	0.496±0.004	0.522±0.005
	2	0.640±0.127	0.508±0.004	0.503±0.002
	4	0.513±0.011	0.504±0.005	0.526±0.018
	24	0.518±0.009	0.505±0.011	0.553±0.026
NZ44	0	0.504±0.001	0.494±0.002	0.511±0.001
	2	0.615±0.225	0.506±0.010	0.501±0.003
	4	0.503±0.003	0.500±0.002	0.500±0.001
	24	0.568±0.023	0.505±0.003	0.525±0.010
GB15	0	0.502±0.002	0.494±0.003	0.569±0.021
	2	0.503±0.003	0.506±0.002	0.511±0.003
	4	0.509±0.009	0.515±0.004	0.591±0.029
	24	0.511±0.004	0.523±0.011	0.491±0.001
AD22	0	0.502±0.002	0.490±0.006	0.514±0.003
	2	0.747±0.197	0.574±0.134	0.507±0.005
	4	0.502±0.002	0.502±0.002	0.541±0.010
	24	0.624±0.051	0.500±0.001	0.489±0.009
NZ3	0	0.510±0.003	0.500±0.008	0.523±0.004
	2	0.491±0.004	0.527±0.048	0.510±0.005
	4	0.503±0.001	0.501±0.003	0.572±0.058
	24	0.543±0.019	0.506±0.008	0.505±0.068

Table 1. Continued.

Codes of isolates	Time of what (h)	Gastric pH		
		2	3	4
		Mean OD at 630 nm		
AD6	0	0.510±0.005	0.422±0.001	0.517±0.001
	2	0.495±0.006	0.596±0.115	0.523±0.006
	4	0.509±0.001	0.502±0.002	0.528±0.028
	24	0.667±0.108	0.510±0.003	1.352±0.063
NZ26	0	0.511±0.007	0.492±0.010	0.514±0.003
	2	0.489±0.001	0.498±0.003	0.510±0.009
	4	0.505±0.002	0.502±0.001	0.533±0.020
	24	0.726±0.094	0.501±0.002	0.870±0.058
BB26	0	0.507±0.003	0.725±0.034	0.511±0.006
	2	0.506±0.003	0.588±0.044	0.511±0.005
	4	0.512±0.004	0.502±0.005	0.510±0.001
	24	0.511±0.006	0.741±0.057	0.519±0.007
DZ9	0	0.519±0.007	0.503±0.008	0.546±0.027
	2	0.515±0.017	0.521±0.019	0.521±0.019
	4	0.504±0.010	0.502±0.001	0.524±0.012
	24	0.522±0.012	0.512±0.005	0.804±0.112
G4	0	0.520±0.018	0.491±0.004	0.546±0.053
	2	0.508±0.012	0.671±0.088	0.504±0.002
	4	0.506±0.005	0.501±0.001	0.512±0.008
	24	0.640±0.065	0.501±0.002	0.507±0.008
DZ5	0	0.508±0.001	0.496±0.004	0.522±0.005
	2	0.640±0.127	0.508±0.004	0.503±0.002
	4	0.513±0.011	0.504±0.005	0.526±0.018
	24	0.518±0.009	0.505±0.011	0.553±0.026
NZ44	0	0.504±0.001	0.494±0.002	0.511±0.001
	2	0.615±0.225	0.506±0.010	0.501±0.003
	4	0.503±0.003	0.500±0.002	0.500±0.001
	24	0.568±0.023	0.505±0.003	0.525±0.010
GB15	0	0.502±0.002	0.494±0.003	0.569±0.021
	2	0.503±0.003	0.506±0.002	0.511±0.003
	4	0.509±0.009	0.515±0.004	0.591±0.029
	24	0.511±0.004	0.523±0.011	0.491±0.001
AD22	0	0.502±0.002	0.490±0.006	0.514±0.003
	2	0.747±0.197	0.574±0.134	0.507±0.005
	4	0.502±0.002	0.502±0.002	0.541±0.010
	24	0.624±0.051	0.500±0.001	0.489±0.009
NZ3	0	0.510±0.003	0.500±0.008	0.523±0.004
	2	0.491±0.004	0.527±0.048	0.510±0.005
	4	0.503±0.001	0.501±0.003	0.572±0.058
	24	0.543±0.019	0.506±0.008	0.505±0.068

Table 1. Continued.

Codes of isolates	Time of what (h)	Gastric pH		
		2	3	4
		Mean OD at 630 nm		
AD17	0	0.506±0.002	0.548±0.007	0.529±0.009
	2	0.514±0.009	0.534±0.008	0.509±0.008
	4	0.515±0.009	0.502±0.004	0.544±0.029
	24	0.527±0.002	0.663±0.103	0.502±0.006
AD29	0	0.513±0.007	0.487±0.001	0.513±0.007
	2	0.495±0.007	0.531±0.017	0.505±0.002
	4	0.519±0.022	0.509±0.004	0.512±0.006
	24	0.532±0.016	0.501±0.003	0.520±0.009
BB3	0	0.511±0.005	0.532±0.012	0.534±0.003
	2	0.506±0.003	0.544±0.015	0.521±0.002
	4	0.524±0.027	0.507±0.002	0.526±0.005
	24	0.521±0.006	0.568±0.017	0.524±0.010
BB31	0	0.509±0.004	0.509±0.009	0.520±0.004
	2	0.509±0.004	0.526±0.016	0.511±0.005
	4	0.516±0.003	0.515±0.014	0.517±0.005
	24	0.526±0.007	0.644±0.076	0.523±0.007
BB50	0	0.500±0.001	0.495±0.004	0.518±0.017
	2	0.507±0.005	0.511±0.008	0.503±0.002
	4	0.500±0.002	0.503±0.005	0.501±0.002
	24	0.505±0.003	0.545±0.019	0.510±0.005
BB60	0	0.496±0.001	0.493±0.003	0.525±0.028
	2	0.505±0.002	0.504±0.002	0.540±0.070
	4	0.498±0.001	0.514±0.002	0.500±0.007
	24	0.506±0.003	0.523±0.012	0.511±0.009
BB61	0	0.513±0.005	0.547±0.006	0.522±0.002
	2	0.506±0.002	0.531±0.003	0.524±0.009
	4	0.514±0.004	0.503±0.002	0.517±0.016
	24	0.528±0.004	0.631±0.058	0.538±0.011
BB64	0	0.502±0.004	0.500±0.010	0.504±0.003
	2	0.510±0.007	0.521±0.007	0.509±0.009
	4	0.511±0.012	0.507±0.005	0.502±0.005
	24	0.513±0.006	0.583±0.023	0.509±0.003
BB7	0	0.503±0.004	0.496±0.002	0.518±0.003
	2	0.502±0.001	0.506±0.003	0.502±0.001
	4	0.506±0.002	0.508±0.004	0.504±0.002
	24	0.511±0.001	0.533±0.017	0.500±0.012
DZ1	0	0.509±0.008	0.528±0.022	0.507±0.003
	2	0.502±0.002	0.508±0.010	0.500±0.002
	4	0.502±0.004	0.509±0.008	0.505±0.005
	24	0.505±0.001	0.520±0.010	0.503±0.001

Table 1. Continued.

Codes of isolates	Time of what (h)	Gastric pH		
		2	3	4
		Mean OD at 630 nm		
DZ13	0	0.511±0.001	0.494±0.018	0.520±0.003
	2	0.508±0.032	0.504±0.002	0.506±0.003
	4	0.506±0.002	0.505±0.002	0.561±0.013
	24	0.575±0.057	0.502±0.001	0.766±0.097
G19	0	0.508±0.002	0.487±0.004	0.520±0.002
	2	0.492±0.001	0.506±0.002	0.504±0.001
	4	0.510±0.006	0.509±0.003	0.519±0.006
	24	0.570±0.017	0.501±0.004	0.515±0.006
G23	0	0.501±0.003	0.498±0.003	0.517±0.003
	2	0.504±0.002	0.504±0.003	0.509±0.009
	4	0.503±0.002	0.502±0.001	0.505±0.004
	24	0.509±0.003	0.578±0.059	0.508±0.004
G25	0	0.501±0.001	0.496±0.003	0.508±0.006
	2	0.503±0.002	0.510±0.005	0.502±0.002
	4	0.511±0.011	0.508±0.006	0.504±0.003
	24	0.510±0.002	0.536±0.012	0.499±0.008
G27	0	0.502±0.002	0.512±0.003	0.519±0.003
	2	0.508±0.006	0.515±0.013	0.510±0.006
	4	0.503±0.004	0.514±0.008	0.512±0.003
	24	0.509±0.006	0.578±0.018	0.526±0.009
G37	0	0.504±0.001	0.502±0.006	0.511±0.001
	2	0.496±0.005	0.570±0.063	0.501±0.002
	4	0.500±0.002	0.507±0.002	0.514±0.008
	24	0.542±0.008	0.503±0.002	0.528±0.003
NZ39	0	0.510±0.004	0.502±0.009	0.520±0.002
	2	0.494±0.001	0.510±0.007	0.510±0.010
	4	0.512±0.004	0.503±0.002	0.528±0.004
	24	0.583±0.014	0.515±0.005	0.670±0.056

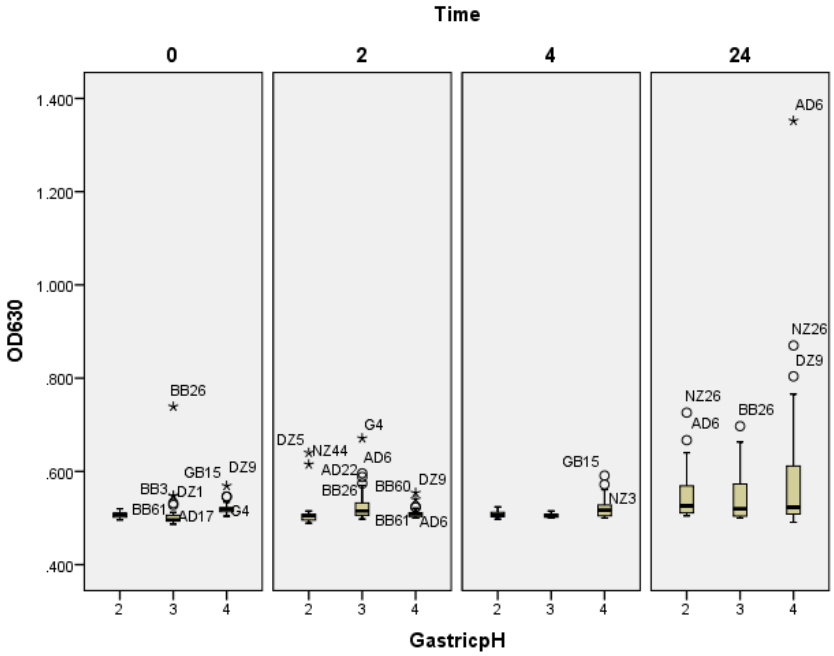

Figure 3. Gastric acid pH interaction effects of probiotic lactic acid bacterial strains with incubation time. The highest tolerance of gastric acid was observed for isolate AD6 at pH 4, 24 h of incubation followed by NZ26 at an absorbance of 630 nm. The pH and time interaction effects of the selected strains varied among the probiotic bacterial isolates.

3.3.2. Bile salt tolerances test

The bile salt tolerance efficiency of twenty-seven (27) selected probiotic lactic acid bacterial strains are indicated in Table 2. Of the twenty-seven (27) probiotic strains isolated, G25 (OD = 0.733 ± 0.103) isolate was able to tolerate 2% of bile salt at 2 h of incubation time. Four isolates, namely, DZ3 (OD = 0.578 ± 0.103), G37 (OD = 0.657 ± 0.046), AD22 (OD = 0.683 ± 0.072) and NZ3 (OD = 0.694 ± 0.070) showed a significant tolerance of 1% of bile salt concentration at 24 h of incubation whereas strains GB 15 (OD = 0.668 ± 0.044), BB7 (OD = 0.595 ± 0.093) and BB50 (OD = 0.681 ± 0.073) tolerate (2%, 24h of incubation at an absorbance of 630 nm). In the present study, most of the probiotic strains were tolerant and survived different bile salt concentrations.

Table 2. Bile salt tolerance efficiency of probiotic lactic acid bacterial isolates.

Codes of isolates	Time	Bile salt concentration			
		0.3%	1%	1.5%	2%
		Mean OD at 630 nm			
G25	0	0.500±0.001	0.529±0.008	0.502±0.005	0.505±0.003
	2	0.503±0.006	0.503±0.004	0.495±0.004	0.733±0.103
	4	0.505±0.003	0.491±0.001	0.495±0.015	0.533±0.005
	24	0.513±0.001	0.495±0.002	0.519±0.033	0.519±0.008
BB50	0	0.507±0.004	0.509±0.003	0.507±0.004	0.509±0.003
	2	0.498±0.004	0.503±0.002	0.502±0.008	0.531±0.012
	4	0.501±0.004	0.493±0.000	0.507±0.005	0.527±0.001
	24	0.530±0.007	0.500±0.006	0.497±0.011	0.681±0.073
NZ3	0	0.515±0.002	0.506±0.007	0.505±0.002	0.506±0.004
	2	0.528±0.049	0.503±0.005	0.497±0.001	0.501±0.004
	4	0.511±0.005	0.549±0.011	0.500±0.007	0.502±0.001
	24	0.487±0.004	0.694±0.070	0.033±0.004	0.496±0.003
AD22	0	0.518±0.014	0.515±0.013	0.503±0.005	0.507±0.004
	2	0.513±0.011	0.520±0.000	0.503±0.012	0.501±0.005
	4	0.512±0.005	0.522±0.011	0.510±0.018	0.509±0.006
	24	0.487±0.004	0.683±0.072	0.022±0.004	0.495±0.001
GB15	0	0.503±0.002	0.510±0.006	0.510±0.008	0.499±0.009
	2	0.508±0.013	0.503±0.003	0.495±0.004	0.576±0.086
	4	0.511±0.004	0.495±0.004	0.506±0.015	0.554±0.024
	24	0.529±0.019	0.506±0.011	0.507±0.004	0.668±0.044
G37	0	0.547±0.002	0.522±0.006	0.517±0.007	0.524±0.005
	2	0.537±0.027	0.511±0.005	0.505±0.006	0.517±0.011
	4	0.539±0.009	0.528±0.010	0.528±0.001	0.520±0.014
	24	0.502±0.006	0.657±0.046	0.021±0.009	0.498±0.002
BB7	0	0.491±0.004	0.517±0.001	0.516±0.002	0.512±0.002
	2	0.502±0.010	0.515±0.006	0.498±0.004	0.564±0.020
	4	0.512±0.005	0.496±0.003	0.506±0.003	0.564±0.021
	24	0.519±0.007	0.506±0.002	0.511±0.009	0.595±0.093
G19	0	0.533±0.013	0.531±0.013	0.536±0.006	0.514±0.014
	2	0.536±0.003	0.515±0.004	0.542±0.016	0.526±0.008
	4	0.518±0.005	0.510±0.003	0.583±0.016	0.507±0.001
	24	0.497±0.009	0.509±0.005	0.013±0.007	0.509±0.005
BB61	0	0.501±0.001	0.506±0.001	0.534±0.086	0.508±0.002
	2	0.506±0.006	0.504±0.001	0.494±0.002	0.520±0.005
	4	0.512±0.007	0.493±0.004	0.503±0.008	0.507±0.001
	24	0.518±0.002	0.507±0.007	0.514±0.009	0.507±0.005
DZ13	0	0.507±0.002	0.510±0.012	0.512±0.011	0.504±0.004
	2	0.501±0.004	0.507±0.017	0.510±0.006	0.502±0.001
	4	0.508±0.007	0.506±0.004	0.504±0.003	0.488±0.005
	24	0.578±0.103	0.031±0.003	0.516±0.018	0.510±0.012

Table 2. Continued.

Codes of isolates	Time	Bile salt concentration			
		0.3%	1%	1.5%	0.3%
		Mean OD at 630 nm			
AD17	0	0.506±0.004	0.512±0.005	0.511±0.003	0.505±0.003
	2	0.539±0.015	0.506±0.002	0.506±0.014	0.558±0.039
	4	0.510±0.001	0.505±0.009	0.498±0.001	0.509±0.001
	24	0.517±0.005	0.506±0.017	0.496±0.001	0.500±0.002
AD29	0	0.524±0.017	0.523±0.013	0.502±0.004	0.514±0.007
	2	0.537±0.038	0.506±0.001	0.499±0.005	0.510±0.001
	4	0.509±0.003	0.502±0.001	0.515±0.008	0.505±0.003
	24	0.525±0.035	0.504±0.003	0.018±0.011	0.508±0.007
AD6	0	0.516±0.007	0.500±0.004	0.500±0.002	0.492±0.004
	2	0.507±0.004	0.503±0.006	0.489±0.001	0.497±0.001
	4	0.513±0.002	0.504±0.002	0.510±0.013	0.503±0.000
	24	0.479±0.007	0.538±0.017	0.028±0.001	0.508±0.011
BB26	0	0.511±0.060	0.508±0.002	0.535±0.027	0.503±0.001
	2	0.513±0.014	0.515±0.007	0.501±0.016	0.518±0.008
	4	0.514±0.009	0.495±0.001	0.497±0.004	0.508±0.002
	24	0.519±0.002	0.499±0.001	0.503±0.003	0.516±0.007
BB3	0	0.499±0.002	0.507±0.004	0.502±0.001	0.503±0.001
	2	0.501±0.004	0.501±0.001	0.499±0.006	0.538±0.012
	4	0.507±0.001	0.489±0.004	0.515±0.012	0.528±0.011
	24	0.523±0.002	0.498±0.001	0.500±0.002	0.563±0.045
BB31	0	0.505±0.003	0.506±0.001	0.501±0.010	0.506±0.003
	2	0.517±0.003	0.542±0.047	0.492±0.002	0.525±0.021
	4	0.507±0.004	0.497±0.003	0.520±0.021	0.505±0.002
	24	0.523±0.006	0.516±0.012	0.522±0.021	0.502±0.002
BB60	0	0.496±0.001	0.512±0.004	0.515±0.007	0.506±0.004
	2	0.510±0.006	0.509±0.005	0.536±0.067	0.545±0.024
	4	0.508±0.003	0.494±0.003	0.509±0.013	0.520±0.002
	24	0.517±0.010	0.505±0.018	0.500±0.003	0.515±0.004
BB64	0	0.510±0.002	0.522±0.015	0.512±±0.004	0.509±0.005
	2	0.523±0.003	0.508±0.002	0.535±0.045	0.544±0.024
	4	0.504±0.001	0.498±0.004	0.498±0.004	0.508±0.004
	24	0.521±0.002	0.499±0.001	0.497±0.001	0.515±0.012
DZ1	0	0.499±0.002	0.514±0.010	0.504±0.004	0.506±0.002
	2	0.522±0.003	0.506±0.002	0.508±0.014	0.520±0.007
	4	0.509±0.002	0.507±0.021	0.502±0.004	0.547±0.011
	24	0.519±0.003	0.499±0.005	0.508±0.006	0.575±0.039
DZ5	0	0.522±0.009	0.529±0.016	0.509±0.004	0.523±0.008
	2	0.5080±.003	0.502±0.005	0.516±0.001	0.514±0.003
	4	0.516±0.003	0.502±0.001	0.527±0.007	0.510±0.002
	24	0.488±0.010	0.502±0.003	0.007±0.002	0.495±0.005

Table 2. Continued.

Codes of isolates	Time	Bile salt concentration			
		0.3%	1%	1.5%	0.3%
		Mean OD at 630 nm			
DZ9	0	0.572±0.021	0.551±0.008	0.565±0.025	0.510±0.005
	2	0.528±0.013	0.521±0.002	0.535±0.016	0.517±0.003
	4	0.554±0.025	0.521±0.005	0.539±0.018	0.511±0.001
	24	0.501±0.009	0.507±0.007	0.0310±0.033	0.507±0.007
G23	0	0.504±0.004	0.530±0.006	0.506±0.002	0.506±0.002
	2	0.505±0.006	0.504±0.003	0.489±0.001	0.507±0.000
	4	0.502±0.003	0.495±0.005	0.497±0.004	0.512±0.003
	24	0.514±0.006	0.500±0.006	0.502±0.006	0.548±0.017
G27	0	0.500±0.002	0.500±0.002	0.506±0.004	0.522±0.016
	2	0.504±0.001	0.499±0.014	0.506±0.005	0.526±0.016
	4	0.516±0.025	0.515±0.012	0.490±0.004	0.531±0.036
	24	0.500±0.003	0.527±0.009	0.511±0.015	0.497±0.001
G4	0	0.544±0.003	0.536±0.014	0.516±0.003	0.517±0.011
	2	0.544±0.015	0.535±0.014	0.510±0.002	0.513±0.001
	4	0.533±0.006	0.507±0.010	0.525±0.006	0.508±0.001
	24	0.513±0.014	0.509±0.029	0.022±0.005	0.505±0.008
NZ26	0	0.508±0.006	0.495±0.009	0.512±0.009	0.519±0.022
	2	0.517±0.019	0.511±0.006	0.498±0.001	0.519±0.010
	4	0.512±0.010	0.500±0.003	0.503±0.004	0.505±0.002
	24	0.486±0.012	0.501±0.005	0.034±0.002	0.499±0.004
NZ39	0	0.511±0.002	0.497±0.004	0.497±0.000	0.508±0.005
	2	0.502±0.005	0.501±0.005	0.500±0.002	0.512±0.011
	4	0.506±0.004	0.531±0.004	0.504±0.004	0.503±0.001
	24	0.505±0.025	0.564±0.046	0.033±0.003	0.497±0.003
NZ44	0	0.509±0.003	0.503±0.007	0.498±0.002	0.527±0.031
	2	0.510±0.005	0.516±0.014	0.495±0.002	0.499±0.002
	4	0.504±0.006	0.506±0.013	0.503±0.009	0.506±0.003
	24	0.488±0.003	0.501±0.008	0.013±0.014	0.496±0.005

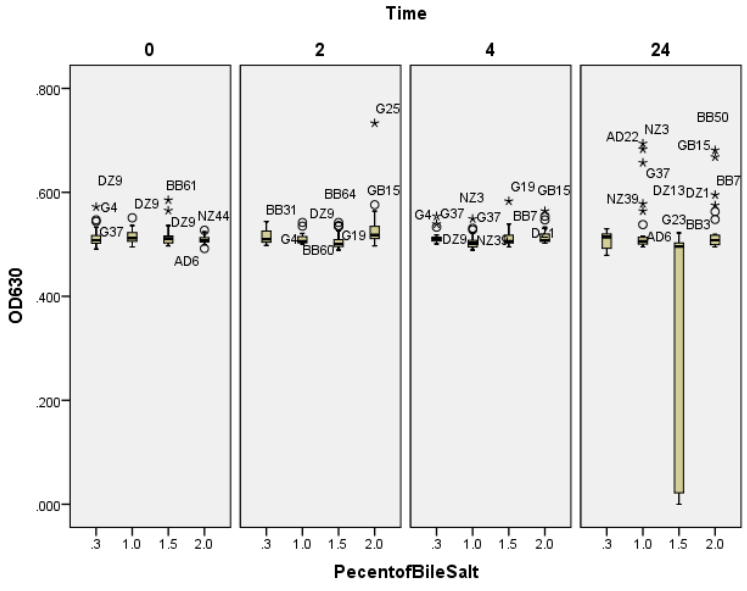

Figure 4. Bile salt concentration tolerance interaction of Lactic acid bacterial strains with incubation time. The highest survival efficiency of bile salt was recorded at a bile salt concentration of 2%, 2h of incubation. Most of the isolates able to grow and survive various bile salt concentrations.

4. Discussion

Twenty-seven isolates showed significant acidification activity, which is 4.2 higher than the other isolates. This is in agreement with the results of Fguiri *et al.* (2016) that *Lactobacillus plantarum* was selected as fast acid producer Lactobacillus isolate from milk. A rapid decrease in pH is essential for coagulation and prevention or reduction of growth of adventitious micro flora in yoghurt production. The fast-acidifying strains are therefore good candidates for dairy fermentation process as primary starter culture while the poor acidification strains can be used as an adjunct culture depending on other properties (Ayad *et al.,* 2004).

Among the 27 isolates, 10 (37%) showed a significance tolerance to various ranges of gastric pH. The tolerance efficiency was varied among the isolated strains. Isolates, namely, AD6 (1.352 ± 0.063), NZ26 (OD = 0.870 ± 0.058) and DZ9 (OD = 0.804 ± 0.112) have shown the highest tolerance of gastric pH (4, 24 h of incubation) compared to the rest probiotic lactic acid bacterial strains at an absorbance of 630 nm. The least gastric tolerance was observed for the isolate AD6 (OD = 0.422 ± 0.001) at a gastric pH of 3 at 0 h of incubation hour. Bacteria that would resist pH values ranging from 2.0 to 8.0 in the gastrointestinal tract if consumed (Hood and Zottola, 1988). Hence, probiotic cultures must survive in the environment with gastric and bile acids, when viable cells go through the gastrointestinal tract. Resisting the pH of 3.0 for 24 h and growing in the medium containing 1,000 ppm of bile acids are considered as standards for acid and bile tolerance of probiotic culture (Itoh, 1992).

A study conducted by Gilliland *et al.* (1984) reported that when a 0.3 absorbance is achieved after at least 2 h of incubation at 37^{o}C in the presence of gastric pH between 1.5 and 4.0, a microorganism can be considered tolerant or resistant to gastric pH. In line with this result, ten out of the 27 isolates tested can be considered tolerant to gastric pH. The highest absorbance was recorded for isolate AD6 (OD = 1.352 ± 0.063) at a pH of 4, 24h of incubation at 37 ^{o}C. However, survival of bacterial strains in human gastric juice is a more accurate indication of the ability of strains to survive passage through the stomach (Draser *et al.,*1969). Similarly, Arokiyamary and Sivakumaar (2011) indicated that lactic acid bacteria isolated from different dairy products were used as a potential probiotic and able to survive in acidic environment (pH = 4 to 6.5). On the other hand, a study conducted by Lee and Salminen (1995) revealed that the LAB survival in low pH is very important for bearing initial stress in the stomach at the application level because, when lactic acid bacteria enters the human body, the first constraint is gastric acid with very low pH level around 2-3. The result of this study showed that probiotic lactic acid bacterial isolates are able to tolerate gastric pH of 2, 3 and 4.

The pH and time interaction effects of the selected strains varied among the probiotic bacterial isolates. The highest tolerance of gastric acid was observed for isolate AD6 at pH 4, 24 h of incubation followed by NZ26 at an absorbance of 630 nm (Figure 3). The effect of acidity on the viability of the isolates was assessed by adjusting the growth medium to different pH values (2, 3 and 4). The present results suggest that probiotic lactic acid bacterial isolates could successfully transit the human stomach and may be capable of reaching the intestinal environment and functioning effectively therein.

Bile salt tolerance is one of the selection criteria whether certain microbes have potentially probiotic function or not presenting the potential of using lactic acid bacteria as effective probiotics it is generally considered necessary to evaluate their ability to resist the effects of bile acids (Goldin *et al.,* 1992). Of the twenty-seven probiotic strains isolated, G25 (OD = 0.733 ± 0.103) isolate was able to tolerate 2% (w/v) of bile salt at 2h of incubation time. Four isolates DZ3 (OD = 0.578 ± 0.103), G37 (OD = 0.657 ± 0.046), AD22 (OD = 0.683 ± 0.072) and NZ3 (OD = 0.694 ± 0.070) showed a significance tolerance of 1% (w/v) of bile salt concentration at 24h of incubation whereas strains GB 15 (OD = 0.668 ± 0.044), BB7 (OD = 0.595 ± 0.093) and BB50 (OD = 0.681 ± 0.073) tolerate 2% (w/v), 24h of incubation at an absorbance of 630 nm). In similar study, Houque *et al.* (2010) studied Lactobacillus sp. isolated four isolates from yogurts and found that all the isolates were able to tolerate bile acid at the rate of 2%. In a similar study, Behboud *et al.* (2011) reported in indicated that resistance to bile salts is considered an important parameter for selecting probiotic strains. A concentration of 0.15–0.3% (w/v) of bile salt has been recommended as a suitable concentration for selecting probiotic bacteria for human use.

In a similar study conducted by Torshizi *et al.* (2008), the survival at bile salt condition is one of the main criteria for in vitro selection of potentially probiotic bacteria and critical points for the microbes. Because some of lactic acid bacteria are able to survive at bile salt condition. Hydrolyses of bile salt decreases the toxic effect of the bile salt to the lactic acid bacteria. In the current study, most lactic acid bacteria isolates are able to survive bile salt. The highest survival efficiency of bile salt was recorded at a bile salt concentration of 2% (w/v), 2h of incubation. Most of the isolates were able to grow and survive various bile salt

concentrations (Figure 4). The high activity of bile salt hydrolysed in lumen of intestine could reduce bile salt conjugation ability to break down lipid (De Smet *et al.,* 1995). Bile salt hydrolytic activity may contribute to the resistance of lactic acid bacteria to the toxicity of conjugated bile salts in the duodenum and therefore is an important colonization factor (Shaikh and Shah, 2013). This may explain the variation recorded among the tested strains in this study. Finally, the present study showed that traditional dairy products are excellent sources of probiotic lactic acid bacteria with the ability to tolerate various gastric and bile salt stress. The isolated strains exhibited an excellent quality of gastric and bile salt tolerance efficiency. In the present study, most of the probiotic strains tolerated and survived different bile salt concentrations.

5. Conclusion

The results obtained in the present study have demonstrated that raw milk and yoghurt contained several groups of probiotic lactic acid bacteria. The findings revealed that naturally occurring lactic acid bacteria isolated from dairy products have the potential for probiotic applications in the dairy industry in the country. The results also suggest that the lactic acid bacterial strains can be selected as good probiotic candidates. Based on the finding of the present study further studies such as molecular characterization, adherence to the alimentary canal, antibiotic resistance and strain stability of the lactic acid bacterial isolates should be conducted. Studies should continue on indigenous diary fermentation and attempts should be made to undertake controlled fermentation studies with potent mixed starter culture with high probiotic functions and optimise the fermentation process conditions. This would result in consistent product with excellent organoleptic properties and keeping good quality of dairy products.

6. Acknowledgements

The authors thank the Ethiopian Institute of Agricultural Research and National Agricultural Biotechnology Research Centre for funding the research.

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