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# Screening of isolated potential probiotic lactic acid bacteria for cholesterol lowering property and bile salt hydrolase activity

**M.G. Shehata [a,*], S.A. El Sohaimy [a], Malak A. El-Sahn [b], M.M. Youssef [b]**

[a] *Food Technology Department, Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Application, Alexandria, Egypt*

[b] *Food Science and Technology Dept., Fac. of Agric., Alexandria Univ., 21545 El-Shatby, Alexandria, Egypt*

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**Abstract** A total of 142 isolates of lactic acid bacteria (LAB) were isolated from dairy and non-dairy sources. The LAB isolates were screened for antimicrobial activity. Out of 142 isolates only 68 isolates exhibited antimicrobial activity. Among these isolates, nine showed wide spectrum antimicrobial activity as well as good bile salt, acid and phenol tolerance. Seven isolates of the latter ones showed more than 20% cholesterol reduction and an observed bile salt hydrolase (BSH) activity. The promising isolates were identified using phenotypic, biochemical and genetic methods.



## Introduction

In recent years, different investigations support the importance of probiotics as apart of healthy diet for humans and animals and as a way to provide a natural, safe and effective barrier against microbial infections (Angmo et al., 2016; Oh and Jung, 2015). According to the definition by the World Health Organization (WHO), probiotics are "live microbial food supplements which, when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). Among the usually used microorganisms, lactic acid bacteria (LAB) are regarded as a major group of probiotic bacteria (Collins and Gibson, 1999). They are non-pathogenic, technologically suitable for industrial processes, acid tolerance, bile tolerance and produce antimicrobial substances (Mojgani et al., 2015). They are classified as generally recognized as safe (GRAS) microorganisms because of their long and safe use as starter cultures in fermented products.

Nowadays, most probiotic bacteria are belonging to the genera *Lactobacillus* and *Bifidobacterium* (Prasad et al., 1998). However, species belonging to the genera *Lactococcus*, *Enterococcus* and *Saccharomyces* (Salminen and von Wright, 1998; Sanders and in't Veld, 1999) are also considered as probiotic microorganisms.

According to the guidelines for the evaluation of probiotics in food reported by a Joint FAO/WHO working group (Vijaya et al., 2015), two of the currently most widely used *in vitro* tests are resistance to gastric acidity and bile salts, as based on both survival and growth studies. Other functional properties used

* Corresponding author. Tel.: +20 34593420; fax: +20 34593423.
E-mail address: gamalsng@gmail.com (M.G. Shehata).

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to characterize probiotics are the production of antimicrobial compounds and cholesterol removal (Park et al., 2007; Xie et al., 2015). The mechanism through which probiotics may antagonize pathogens involves production of antimicrobial compounds such as lactic acid, acetic acid, hydrogen peroxide and bacteriocins.

Certain studies showed that among the other effects of probiotic include, consumption of lactic acid bacteria reduced carriage of pathogens microorganism, decreased certain risk factors for coronary artery disease, and resulted in a dose-dependent reduction in the symptoms of Irritable bowel syndrome (Vries et al., 2006). It seems that, in the research for strains with probiotic potential, food might also be a good source of suitable isolates for finding new probiotic strains for functional food products. Several probiotics bacteria are found to produce bile salt hydrolase (BSH) that helps to reduce serum cholesterol (Miremadi et al., 2014) and hence BSH activity is also considered as an additional criterion for the selection of probiotics. The aim of the present study was to isolate, identify and screen for potential probiotic lactic acid bacteria with high cholesterol capacity and bile salt hydrolase activity.

## Materials and methods

### *Collection of samples*

Samples were collected from the normal habitats of lactic acid bacteria (LAB) such as raw animal milk, fermented foods (*Boza*, *Zabady*, *Rayeb*), cheese (*Ras*, *Kareish*), calves infant faeces and intestinal of marine fish (Table 1). Samples were transported to the laboratory in ice box and stored at ≅ 4 °C.

**Table 1** Isolation sources of lactic acid bacteria.

| Isolation Sources | Number of examined samples | Number of isolates | Location |
|---|---|---|---|
| *Boza* (BO) | 2 | 43 | Manfalut City, Assiut Governorate, Egypt Tanta city, Gharbia Governorate, Egypt |
| Calves infants faeces (F) | 2 | 10 | Private farm, Damanhour city, Beheira Governorate, Egypt |
| *karish* cheese (k) | 3 | 20 | Alexanderia, local markets |
| Milk (M) | 2 | 10 | Alexanderia, local markets |
| *Ras* cheese (R) | 3 | 15 | Alexanderia, local markets |
| *Rayeb* Milk (RM) | 2 | 19 | Alexanderia, local markets |
| The intestines of marine fish (I) | 2 | 0 | Alexanderia, local markets |
| *Zabady* (Z) | 4 | 25 | Alexanderia, local markets |
| Total | 20 | 142 | |

### *Isolation of lactic acid bacteria*

Milk samples were incubated at 30 °C; 37 °C; 42 °C, while samples of cheese, *Zabady* and *Rayeb* were cultured in sterilized reconstituted skim milk and incubated until coagulation. Coagulated samples were then streaked on over agar surface of MRS medium (De Man et al., 1960) and were incubated anaerobically at 30 °C, 37 °C or 42 °C for 48 h. *Boza*, calves infant faeces and intestine of marine fish samples, were diluted serially from $10^{-1}$ to $10^{-7,}$ then 0.1 ml aliquot of the higher dilutions ($10^{-4}$ to $10^{-7}$) were spread on to MRS plates and incubated at 30, 37 and 42 °C for 48 h.

White and creamy colonies were picked up randomly and purified by three successive transfers on MRS medium. The cultures were routinely checked for purity by microscopic examination.

The pure cultures were characterized using Gram stain, cell morphology and catalase reaction according to standard procedures (Sharpe, 1979). Gram-positive and catalase-negative isolates were selected and stored at −80 °C in MRS broth plus 28% glycerol (El-Soda et al., 2003). The purified cultures were activated by subculturing twice in MRS broth before use.

### *Antimicrobial activity assays*

#### *Screening of LAB isolates for antimicrobial activity*

The Antimicrobial activity spectrums of cells free supernatants of (LAB) isolates were determined against ten pathogens (Table 2) using spot-on-lawn method (Barefoot and Klenhammer, 1983). LAB isolates were cultivated in MRS for 16-18 h with 1% inoculum then cells were removed from MRS medium by centrifugation (6500*g* for 10 min, 4 °C) to obtain cell free supernatant. Lawns of pathogenic strains were prepared by adding 0.125 ml ($2 \times 10^7$ cell/ml) of 10× diluted overnight culture to 5 ml of corresponding soft agar (Table 2). The contents of the tubes were gently mixed and poured over the surfaces of pre-poured MRS agar plates. Ten microliter of each cell free supernatant was spotted onto the surface of the soft agar plate and after 24 h of incubation, the plates were

**Table 2** Indicator strains and their growth conditions.

| Pathogenic microorganisms | Medium and growth temperature |
|---|---|
| *Bacillus subtilis* DB 100 host | Nutrient Broth, 37 °C |
| *Candida albicans* ATCC MYA-2876 | YPD[a] Broth, 37 °C |
| *Clostridium botulinum* ATCC 3584 | TPGY[b] Broth, 37 °C |
| *Escherichia coli* BA 12296 isolated by dr sobhy | LB[c] Broth, 37 °C |
| *Klebsiella pneumoniae* ATCC12296 | LB[c] Broth, 37 °C |
| *Salmonella senftenberg* ATCC 8400 | Nutrient Broth, 37 °C |
| *Staphylococcus aureus* NCTC 10788 | Nutrient Broth, 37 °C |
| *Staphylococcus epidermidis* | Nutrient Broth, 37 °C |
| *Streptococcus dysgalactiae* subsp. Equisimilis | Nutrient Broth, 37 °C |
| *Streptococcus pyogenes* | Nutrient Broth, 37 °C |

[a] YPD broth: *Yeast peptone dextrose*.
[b] TPGY broth: tryptone–peptone–glucose–yeast extract.
[c] LB broth: Luria-Bertani medium.

checked for the appearance of an inhibition zone. Clear zones around the spots indicate the antimicrobial activity of isolated bacteria.

*Quantification of antimicrobial activity*

Antimicrobial activity in the supernatant was determined by an adaptation of the critical two fold dilution method (Parente et al., 1994). Each tested isolate was subcultured in MRS for 24 h, counted and adjusted to give $10^9$ cell/ml. The culture was used to inoculate (1%) fresh MRS and propagated for 24 h. Supernatant was obtained by centrifugation (9000*g* for 15 min). Serial two-fold dilutions of supernatant were carried out in MRS. Activity was quantified by taking the reciprocal of the highest dilution that exhibited a clear zone of inhibition and was expressed as activity units (AU) per millilitre of culture media. The titre of the antibacterial substance, in AU/ml, was calculated as (1000/*d*) *D*, where *D* is the dilution factor and *d* is the amount of supernatant in µl (Parente et al., 1994).

*Acid tolerance*

Isolates of LAB were propagated twice in MRS broth (1% v/v) for 20 h at 37 °C before experimental use. The cells from 100 ml MRS culture were harvested by centrifugation (4300*g*, 10 min), and washed three times in phosphate-buffered saline, pH 7.0. Washed cell pellets were then suspended in (1/10) cultivation volume in the same buffer, hence obtaining a 10-fold increase in cell density. To 1 ml of the washed cell suspension, 5 ml of simulated gastric juice and 1.5 ml NaCl (0.5 w/v) were added. Simulated gastric juice was prepared freshly daily by suspending pepsin (3 g/L) in sterile saline (0.5% w/v) and adjusting the pH to 2.0 with concentrated HCl (Charteris et al., 1998). The materials were vortexed for 10 s and incubated at 37 °C for 3 h. Aliquots of 0.1 ml were then removed at constant intervals (0, 1, 2, 3 h) for determination of total viable count. Dilutions were made (up to $10^{-4}$) and cells were plated in duplicate on MRS agar. Plates were incubated at 37 °C for 72 h before enumeration (Charteris et al., 1998).

*Bile tolerance*

Bile containing MRS broth was prepared by the addition of 0.3 (v/v) of bile salt (Bio Basic Canada INC.). The cells from 100 ml (20 h MRS tested culture) were collected by centrifugation (3400*g*, 10 min), washed twice in saline (8.5 g NaCl/L) and resuspended in 10 ml MRS broth. This suspension was inoculated (1%) into MRS broth lacking or containing bile salt. After 0, 1, 2 and 3 h of incubation at 37 °C, viable counts on MRS agar plates and absorbance of the culture at 625 nm were determined (Matijasic and Rogelj, 2000). Experiments of acid and bile tolerance were repeated three times each with duplicate analysis.

*Phenol tolerance*

Phenol tolerance experiments were performed as described by Aswathy et al. (2008) with slight modifications. The overnight cultures of LAB isolates were inoculated (1%) into MRS broth with (0.2 and 0.5% v/v) or without phenol. Bacterial cells in the culture broth were measured by reading the absorbance (A) at 600 nm after 24 h of incubation at 37 °C. The experiments were repeated twice in duplicate.

*Cholesterol assimilation*

Freshly prepared MRS broth, supplemented with 0.3% oxgall (Bio Basic Canada INC.) as bile salt and filter sterilized water soluble cholesterol (100 µg/ml), was inoculated with each isolate at 1% level and incubated anaerobically at 37 °C for 24 h. After incubation period, cells were removed by centrifugation (9000*g* for 15 min) and the remaining cholesterol in the spent broth was determined calorimetrically using *o*-phthalaldehyde method described by Rudel and Morris (1973). One millilitre of the cell- free broth was added to 1 mL of KOH (33% wt/vol) and 2 mL of absolute ethanol, vortexed for 1 min, followed by heating at 37 °C for 15 min. After cooling, 2 mL of distilled water and 3 mL of hexane were added and vortexed for 1 min. One millilitre of the hexane layer was transferred into a glass tube and evaporated in water bath at 65 °C. The residue was immediately dissolved in 2 mL of *o*-phthalaldehyde reagent. After complete mixing, 0.5 mL concentrated sulphuric acid was added and the mixture was vortexed for 1 min. Absorbance was read at 550 nm (T80 UV/Vis spectrometer PG Instruments LDT, United Kingdom) after 10 min. All experiments were replicated twice.

*Screening of probiotic LAB for bile salts hydrolases activity (BSH)*

*Qualitative determination of bile salts hydrolases activity*

The BSH activity was determined as described by Du Toit et al. (2003). The LAB isolates were grown on MRS agar plates containing 0.5% (w/v) taurodeoxycholic acid sodium salt (TDCA; Sigma, USA) and 0.037% calcium chloride. Plates were incubated under anaerobic conditions at 37 °C for 72 h. The precipitation zone surrounding colonies indicated the bile salt hydrolase activity of bacteria.

*Quantitative determination of bile salts hydrolases activity*

The BSH activity was determined by measuring the amount of amino acid liberated from conjugated bile salts, by the probiotic isolates as described by Tanaka et al. (2000) with several modifications. Briefly, from cultures grown for 20 h at 37 °C, cells were harvested by centrifugation at 9700*g* for 15 min, washed twice with 0.1 M sodium phosphate buffer containing 10 mM dithiothreitol (DTT), pH 6.8 and re-suspended in the same buffer to obtain a suspension with an optical absorbance ($A_{600\ nm}$) of 3.0. Cell suspension was sonicated for 60 s. with cooling on ice with two cycles of 16 mm using a sonicator (Sonics and Materials Inc., Vibro cell), followed by centrifugation at 9700*g* for 15 min. The reaction mixture consisted of 180 mL of 0.1 M sodium phosphate buffer, pH 6.0, 10 mL of a 200 mM appropriate conjugated bile salt, 10 mM DTT and 10 mL of cell-free extract. The reaction mixture was incubated at 37 °C for 30 min., then a sample (100 µL) was taken and 200 µL of 15% (w/v) trichloroacetic acid was added to terminate the reaction. The sample was centrifuged (9700*g* for 15 min) and 200 µL of the supernatant was added to 200 µL

of distilled water and 1.9 mL of ninhydrin reagent (5 mg ninhydrin, 1.2 mL glycerol, and 0.7 mL 0.5 M pH 5.5 sodium citrate buffer). The mixture was vortexed and boiled for 14 min. After subsequent cooling, the absorbance at 570 nm was determined using glycine or taurine as standard. One unit of BSH activity (U/ml) was defined as the amount of enzyme that liberated 1 mmol of amino acid from the substrate per min. Protein concentration was determined by the Lowry method (Lowry et al., 1951), with bovine serum albumin (Sigma) as standard. All experiments were repeated twice.

*Identification of the promising LAB isolates*

*Phenotypic characterization*

The promising LAB isolates were phenotyped as described in Bergey's manual of systematic bacteriology (Logan and De Vos, 2009). The following tests were applied: cell morphology; growth at 15, 37 and 45 °C; and growth in MRS containing 2.5%, 4% and 6.5% NaCl. Fermentation patterns were determined using API 50 CHL and API 20 kits (Biomerieux SA, France) according to the manufacturer's instructions.

*Molecular identification*

*Extraction of bacterial DNA*. The DNA extraction and purification from bacterial isolates were carried out according to the procedure described by Cheng and Jiang, 2006. Overnight bacterial cultures were centrifuged individually at 15,000*g* for 10 min. Pellets were washed with 400 µl SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5), then centrifuged at 10,000*g* for 10 min. The pellets were resuspended in TE buffer (Tris-EDTA buffer, pH 8.0), 100 µl tris-saturated phenol (pH 8.0) was added and the suspensions were centrifuged at 10,000*g* for 10 min at 4 °C. Mixtures composed each of 160 µl of the obtained aqueous phase, 40 µl TE buffer and 100 µl chloroform were centrifuged at 10,000*g* for 10 min. The resulting supernatant (100 µl) was mixed with 40 µl TE buffer and 5 µl RNase (10 mg/ml) and incubated at 37 °C for 10 min. Then 100 µl chloroform was added and the mixture was centrifuged at 15,000*g* for 10 min at 4 °C. The purity as well as the yield of DNA in the aqueous phase was assessed spectrophotometrically.

*Polymerase chain reaction amplification*. Universal primers identifying LAB, designed using the invariant region in the 16 s rDNA sequences for LAB (Wang et al., 1996), were obtained from Sigma Scientific Services Co., Germany. The reaction mixture (20 µl) consisted of 5 µl colourless GoTaq® reaction Buffer (5×), 0.25 µl GoTaq® DNA Polymerase (5 u/µl) (Promega, USA), 2.5 µl PCR nucleotide Mix (10 mM), 1 µl of each primer 5/CGTGCCAGCCGCGGTAATACG 3/and 5/GGGTTGCGCTCGTTGCGGGACT TAACCCAACAT 3/) as forward and reverse primers, respectively, 2 µl genomic DNA and 8.25 µl of nuclease-free water.

The PCR amplification was carried out in the thermo cycler PCR (Santa Clara, California, United States) according to the following programme: initial denaturation at 95 °C for 5 min, amplification for 30 cycles [95 °C/40 s (denaturation), 55 °C/40 s (annealing), 72 °C/1 min (extension)], then final extension at 72 °C for 10 min. The products were separated on 1% agarose gel containing ethidium bromide (1 µg/ml), then image was taken using gel documentation system (Syngene Bio Maging, Canada). The DNA marker 100–1500 bp (TAKARA BIO INC., Shiga, Japan) was used as the molecular weight standard.

*Sequencing of DNA*. The DNA sequencing reactions were performed using an automated DNA sequencer. Database searches were performed using the latest release of non-redundant DNA sequence database present at the National Centre for Biotechnology Information (NCBI) website located at: http://www.ncbi.nlm.nih.gov/BLAST (Altschul et al., 1997).

*Statistical analysis*

Data were statistically analysed with CoStat software (version 6.303). One-way analysis of variance was used to study significant difference between means, with significance level at $P = 0.05$.

## Results and discussions

*Screening of LAB isolates for their antimicrobial activity*

One hundred and forty-two LAB were isolated from various sources (Table 1). All isolated bacteria fit the classification of LAB as Gram-positive, catalase negative (Sharpe, 1979). The antimicrobial activity is one of the most important selection criteria for probiotic. The LAB isolates were screened for production of antimicrobial agents against 10 pathogens (Table 2).

Out of 142 LAB isolates only 68 isolates exhibited antagonistic activity with varying degrees. Out of sixty LAB strains isolated from *zabady* and cheese samples, only thirty-eight strains showed inhibitory activity against the tested pathogens. No antagonistic activity could be observed for LAB strains isolated from milk and calves infant faeces. The LAB strains that showed inhibitory activity against more than six of the tested pathogens were isolated from *Boza* and *Rayeb* milk samples. On the other hand, LAB isolates obtained from *zabady*, *Ras* cheese and *Karish* exhibited antagonistic activity only against three or less of the tested pathogens. Indicator pathogens can be organized in descending order according to their sensitivity to the tested isolates as follows: *Escherichia coli* BA 12296 was sensitive to 41 isolates while *Staphylococcus epidermidis, Salmonella senftenberg* ATCC 8400*, Staphylococcus aureus* NCTC 10788*, Streptococcus pyogenes, Klebsiella pneumoniae* ATCC12296*, Candida albicans* ATCC MYA-2876*, Streptococcus dysgalactiae* subsp. *equisimilis*, and *Bacillus subtilis* DB 100 were sensitive to 28, 29, 23, 19, 15, 14, 10, 2 of LAB isolates, respectively.

All isolates with antimicrobial activity against any of the tested pathogens (68 isolates) were selected for quantitative determination of their antimicrobial activities (Table 3). Among 68 isolates, only 9 isolates showed wide spectrum activity against four tested pathogens at least.

Among the nine isolates of LAB, only one isolate (RM39) showed strong activity of 1600 AU/ml against *Klebsiella pneumoniae* ATCC12296 *while, four isolates exhibited inhibitory activity of 800* AU/ml against each of *Escherichia coli* (BO51); *Streptococcus pyogenes* (RM28); *Staphylococcus aureus* NCTC 10788 (BO3, RM3), *Salmonella senftenberg* ATCC

**Table 3** Antimicrobial activities of cell-free supernatants of 68 selected LAB isolates against various pathogens.[a]

| Isolate no | Sym | Sources | Incubation T (C) | Antimicrobial activity against pathogens expressed in AU/ml[c] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Bac* | *Cand.* | *Clo.* | *E. coli* | *Kleb.* | *Sal.* | *St.au.* | *St.epi* | *St.Py.* | *St. dy.* |
| 1. | [d]BO3 | BO[b] | 30 | **0** | **0** | **0** | **400** | **0** | **400** | **800** | **200** | **200** | **400** |
| 2. | BO4 | BO | 30 | **0** | **0** | **0** | **200** | **0** | **200** | **0** | **0** | **0** | **0** |
| 3. | BO5 | BO | 30 | 0 | 0 | **0** | **200** | **0** | **0** | **0** | **200** | **0** | **0** |
| 4. | BO6 | BO | 30 | **0** | **0** | **0** | **200** | **0** | **0** | **0** | **0** | **0** | **0** |
| 5. | BO7 | BO | 30 | **0** | **0** | **0** | **400** | **800** | **200** | **0** | **0** | **0** | **0** |
| 6. | BO8 | BO | 30 | **0** | **0** | **0** | **200** | **0** | **200** | **0** | **200** | **0** | **0** |
| 7. | BO12 | BO | 30 | **0** | **0** | **0** | **200** | **0** | **200** | **0** | **200** | **0** | **0** |
| 8. | BO22 | BO | 37 | **0** | **0** | **0** | **200** | **0** | **200** | **0** | **200** | **0** | **0** |
| 9. | BO24 | BO | 37 | **0** | **0** | **0** | **200** | **0** | **200** | **0** | **200** | **0** | **0** |
| 10. | BO27 | BO | 37 | **0** | **0** | **0** | **200** | **0** | **200** | **0** | **200** | **0** | **0** |
| 11. | BO29 | BO | 37 | **0** | **0** | **0** | **200** | **0** | **200** | **0** | **200** | **0** | **0** |
| 12. | BO30 | BO | 37 | **0** | **0** | **0** | **200** | **0** | **200** | **0** | **200** | **0** | **0** |
| 13. | BO31 | BO | 37 | **0** | **0** | **0** | **200** | **0** | **200** | **0** | **200** | **0** | **0** |
| 14 | BO33 | BO | 37 | **0** | **0** | **0** | **200** | **400** | **400** | **0** | **0** | **0** | **0** |
| 15. | [b]BO34 | BO | 37 | **200** | **0** | **0** | **800** | **800** | **400** | **0** | **0** | **0** | **0** |
| 16. | [d]BO35 | BO | 37 | **0** | **0** | **0** | **800** | **200** | **800** | **200** | **200** | **0** | **200** |
| 17. | BO36 | BO | 37 | **0** | **0** | **0** | **400** | **0** | **0** | **200** | **400** | **0** | **0** |
| 18. | [d]BO37 | BO | 37 | **800** | **0** | **0** | **400** | **0** | **200** | **200** | **800** | **400** | **400** |
| 19. | BO42 | BO | 42 | **0** | **0** | **0** | **400** | **200** | **0** | **0** | **400** | **0** | **0** |
| 20. | BO44 | BO | 42 | **0** | **0** | **0** | **0** | **200** | **400** | **0** | **0** | **400** | **0** |
| 21. | BO45 | BO | 42 | **0** | **0** | **0** | **200** | **0** | **200** | **200** | **0** | **0** | **0** |
| 22. | BO46 | BO | 42 | **0** | **0** | **0** | **200** | **0** | **200** | **200** | **0** | **0** | **0** |
| 23. | BO50 | BO | 42 | **0** | **0** | **0** | **0** | **0** | **200** | **200** | **0** | **200** | **0** |
| 24. | [d]BO51 | BO | 42 | **0** | **400** | **0** | **800** | **200** | **200** | **200** | **400** | **200** | **400** |
| 25. | [d]BO52 | BO | 42 | **0** | **0** | **0** | **400** | **400** | **0** | **0** | **400** | **0** | **400** |
| 26. | Z1 | Z[b] | 30 | **0** | **0** | **200** | **0** | **0** | **0** | **200** | **0** | **200** | **0** |
| 27. | Z2 | Z | 30 | **0** | **0** | **200** | **0** | **0** | **0** | **200** | **0** | **200** | **0** |
| 28. | Z3 | Z | 30 | **0** | **0** | **0** | **0** | **200** | **0** | **0** | **200** | **200** | **0** |
| 29. | Z4 | Z | 30 | **0** | **0** | **0** | **200** | **0** | **0** | **200** | **0** | **200** | **0** |
| 30. | Z5 | Z | 30 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **200** | **0** |
| 31. | Z8 | Z | 30 | **0** | **0** | **0** | **0** | **200** | **0** | **0** | **0** | **200** | **0** |
| 32. | Z9 | Z | 30 | **0** | **0** | **0** | **0** | **200** | **0** | **0** | **0** | **0** | **200** |
| 33. | Z22 | Z | 37 | **0** | **200** | **0** | **400** | **0** | **0** | **0** | **0** | **0** | **0** |
| 34. | Z23 | Z | 37 | **0** | **0** | **0** | **0** | **200** | **200** | **0** | **0** | **0** | **200** |
| 35. | Z25 | Z | 37 | **0** | **0** | **0** | **0** | **0** | **400** | **400** | **0** | **0** | **0** |
| 36. | Z27 | Z | 37 | **0** | **0** | **0** | **200** | **0** | **0** | **200** | **0** | **0** | **0** |
| 37. | Z28 | Z | 37 | **0** | **0** | **0** | **0** | **200** | **0** | **200** | **400** | **0** | **0** |
| 38. | Z39 | Z | 37 | **0** | **200** | **0** | **0** | **0** | **0** | **200** | **400** | **0** | **0** |
| 39. | Z41 | Z | 42 | **0** | **0** | **0** | **0** | **0** | **200** | **200** | **200** | **0** | **0** |
| 40. | Z42 | Z | 42 | **0** | **0** | **0** | **200** | **0** | **0** | **200** | **200** | **0** | **0** |
| 41. | R20 | R[b] | 37 | **0** | **0** | **0** | **200** | **0** | **0** | **0** | **200** | **0** | **0** |
| 42. | R22 | R | 37 | **0** | **0** | **0** | **200** | **0** | **0** | **200** | **0** | **0** | **0** |
| 43. | R24 | R | 37 | **0** | **0** | **0** | **200** | **0** | **0** | **200** | **0** | **0** | **0** |
| 44. | R25 | R | 37 | **0** | **0** | **0** | **0** | **200** | **200** | **0** | **400** | **0** | **0** |
| 45. | R26 | R | 37 | **0** | **0** | **0** | **0** | **0** | **200** | **0** | **0** | **200** | **0** |
| 46. | R27 | R | 37 | **0** | **200** | **0** | **0** | **0** | **0** | **200** | **0** | **0** | **0** |
| 47. | R28 | R | 37 | **0** | **200** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** |
| 48. | R34 | R | 37 | **0** | **0** | **0** | **0** | **0** | **0** | **200** | **200** | **0** | **0** |
| 49. | K1 | K[b] | 30 | **0** | **0** | **0** | **200** | **0** | **0** | **200** | **0** | **0** | **0** |
| 50. | K2 | K | 30 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **400** | **0** |
| 51. | K4 | K | 30 | **0** | **0** | **0** | **0** | **0** | **0** | **200** | **200** | **0** | **0** |
| 52. | K5 | K | 30 | **0** | **200** | **0** | **0** | **0** | **0** | **200** | **0** | **400** | **0** |
| 53. | K6 | K | 30 | **0** | **0** | **0** | **200** | **0** | **0** | **0** | **0** | **200** | **0** |
| 54. | K7 | K | 30 | **0** | **0** | **0** | **0** | **0** | **200** | **0** | **0** | **200** | **0** |
| 55. | K8 | K | 30 | **0** | **0** | **0** | **200** | **0** | **0** | **0** | **0** | **0** | **0** |
| 56. | K9 | K | 30 | **0** | **200** | **0** | **0** | **0** | **0** | **400** | **0** | **0** | **0** |
| 57. | K23 | K | 37 | **0** | **200** | **0** | **200** | **0** | **0** | **0** | **0** | **200** | **0** |
| 58. | K24 | K | 37 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **200** |
| 59. | K26 | K | 37 | **0** | **200** | **0** | **0** | **0** | **0** | **0** | **0** | **200** | **0** |
| 60. | K27 | K | 37 | **0** | **200** | **0** | **200** | **0** | **0** | **0** | **0** | **0** | **0** |
| 61. | K40 | K | 42 | **0** | **0** | **0** | **200** | **0** | **0** | **0** | **0** | **0** | **0** |

(*continued on next page*)

**Table 3** (*continued*)

| Isolate no | Sym | Sources | Incubation T (C) | Antimicrobial activity against pathogens expressed in AU/ml[c] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Bac* | *Cand.* | *Clo.* | *E. coli* | *Kleb.* | *Sal.* | *St.au.* | *St.epi* | *St.Py.* | *St. dy.* |
| 62. | K42 | K | 42 | **0** | **200** | **0** | **200** | **0** | **0** | **0** | **0** | **200** | **0** |
| 63. | K43 | K | 42 | **0** | **200** | **0** | **0** | **0** | **0** | **0** | **0** | **200** | **0** |
| 64. | RM1 | RM[b] | 30 | **0** | **0** | **0** | **200** | **200** | **200** | **0** | **0** | **0** | **0** |
| 65. | [d]RM3 | RM | 30 | **0** | **1600** | **0** | **400** | **200** | **0** | **800** | **200** | **200** | **800** |
| 66. | RM26 | RM | 30 | **0** | **0** | **0** | **0** | **200** | **0** | **0** | **200** | **200** | **0** |
| 67. | [d]RM28 | RM | 30 | **0** | **0** | **0** | **400** | **200** | **800** | **200** | **400** | **800** | **0** |
| 68. | [d]RM39 | RM | 30 | **0** | **200** | **0** | **400** | **1600** | **400** | **200** | **400** | **0** | **400** |

[a] *Bac.*: – *Bacillus Subtilis; Cand.*: – *Candida albicans; Clo.*: – *Clostridium botulinum; E. coli*: – *Escherichia coli; Kleb.*: – *Klebsiella pneumoniae; Sal.*: – *Salmonella Senftenberg; St.au.*: – *Staphylococcus aureus; St.epi.*: – *Staphylococcus Epidermidis; Str. pyo.*: – *Streptococcus pyogenes*; *St. dy.*: – *Streptococcus dysgalactiae* subsp. *Equisimilis*.

[b] Sources of isolation: BO: *Boza*; Z: *Zabady*; R: *Ras* cheese; K: *karish* cheese; RM: *Rayeb* Milk.

[c] Activity units/ml cell-free supernatant was calculated according to the following equation: (1000/d) × D ،Where **D** is *the two-fold dilution factor*, **d** is *the amount of supernatant used.*

[d] Isolates had antimicrobial activity against 4 or more pathogenic microorganisms.

**Table 4** Survival of selected lactic acid bacteria isolates under simulated gastric juice conditions at 37 °C.

| Isolates code | Mean of viable count ($\log_{10}$ CFU $ml^{-1}$) ± SD[*] | | | | Surviving percentage (%) |
|---|---|---|---|---|---|
| | Time of exposure (h) | | | | |
| | 0 | 1 | 2 | 3 | |
| **Bo 3** | **8.29** ± 0.41[a] | **7.80** ± 0.14[bcd] | **7.04** ± 0.20[cd] | **6.32** ± 0.25[d] | **76.2** |
| **Bo 34** | **8.31** ± 0.29[a] | **7.91** ± 0.03[bc] | **7.21** ± 0.16[bc] | **6.37** ± 0.08[d] | **76.6** |
| **Bo 35** | **8.17** ± 0.317[a] | **7.60** ± 0.23[cde] | **7.36** ± 0.27[bc] | **7.22** ± 0.28[ab] | **88.3** |
| **Bo 37** | **8.55** ± 0.22[a] | **7.86** ± 0.04[bcd] | **7.51** ± 0.14[ab] | **7.33** ± 0.13[a] | **85.7** |
| **Bo 51** | **8.37** ± 0.25[a] | **7.55** ± 0.07[de] | **7.13** ± 0.10[cd] | **6.45** ± 0.38[cd] | **77** |
| **Bo 52** | **8.37** ± 0.44[a] | **8.33** ± 0.45[a] | **7.57** ± 0.17[ab] | **6.59** ± 0.24[cd] | **78.7** |
| **RM 3** | **8.35** ± 0.34[a] | **7.28** ± 0.17[e] | **6.81** ± 0.19[de] | **6.81** ± 0.19[bc] | **81.5** |
| **RM 28** | **8.41** ± 0.44[a] | **7.58** ± 0.13[cde] | **6.60** ± 0.41[e] | **5.72** ± 0.27[e] | **68** |
| **RM 39** | **8.37** ± 0.28[a] | **8.03** ± 0.20[ab] | **7.85** ± 0.09[a] | **6.48** ± 0.30[cd] | **77.4** |

[abcde]Means in the same column followed by different superscript letters are significantly different ($P < 0.05$).

BO: – Boza & RM: – *Rayeb* Milk.

[*] Results are expressed as mean ± SD, and each value is the average of three experiments and each was carried out in duplicate.

8400 (RM28). Growth of pathogens is inhibited by the production of antimicrobial compounds such as organic acids, hydrogen peroxide, diacetyl and bacteriocins by LAB as well as their competition for nutrients (Bezkorvainy, 2001; Tambekar et al., 2009).The nine promising LAB isolates were examined for further probiotic criteria such as bile, acid and phenol tolerance as well as their cholesterol removal capacities.

*Tolerance to acid and bile*

As probiotics are usually administrated orally, they must have the ability to survive passage through the stomach and small intestine. Therefore, resistance to the low pH of the gastric juice in the stomach and the bile salt in the small intestine is one of the important selection criteria for probiotic (Olejnik et al., 2005). In the present study, all the selected LAB isolates were able to survive simulated gastric juice at pH 2 after 3 h of incubation (Table 4). They retained varying levels (68–88.3%) of viability. The highest survival was for BO35 isolate while the least survival was observed for RM28 isolate.

Acid tolerance of bacteria is important not only for withstanding gastric stresses, but also a prerequisite for their use as dietary adjuncts and enables strains to survive for longer period of time in high acid carrier food without larger reduction in humans (Conway et al., 1987; Prasad et al., 1998).

Tolerance to bile salts is a prerequisite for colonization and metabolic activity of bacteria in small intestine of the host (Havenaar et al., 1992). This will help *Lactobacillus* spp. and *Lactococcus* spp. to reach the small intestine and colon and contribute in balancing the intestinal microflora (Tambekar and Bhutada, 2010). All the tested strains exhibited bile tolerance with varying degrees. Among the tested LAB isolated in the present study, BO34 isolate demonstrated the highest bile salt tolerance followed by BO52 isolate (Table 5).

*Phenol tolerance*

For a strain to be a probiotic, it has to survive the action of toxic metabolites, primarily phenols, produced during the digestion process (Hoier, 1992). Some aromatic amino acids derived from dietary or endogenously produced proteins can be deaminated in the gut by bacteria leading to the formation of phenols which have bacteriostatic properties (Suskovic et al., 1997).

**Table 5** Survival of selected lactic acid bacteria isolates in MRS broth supplemented with 0.3% bile salts after 0, 1, 2 and 3 h at 37 °C.

| Isolates code | Mean of viable count ($\log_{10}$ CFU $ml^{-1}$) ± SD* | | | | Surviving percentage (%) |
|---|---|---|---|---|---|
| | Time of exposure (h) | | | | |
| | 0 | 1 | 2 | 3 | |
| **Bo 34** | **8.05** ± 0.05$^{a}$ | **7.63** ± 0.26$^{ab}$ | **7.04** ± 0.21$^{a}$ | **6.85** ± 0.10$^{ab}$ | **85** |
| **Bo 35** | **7.98** ± 0.43$^{a}$ | **6.77** ± 0.41$^{d}$ | **6.54** ± 0.21$^{c}$ | **5.69** ± 0.32$^{d}$ | **71.3** |
| **Bo 51** | **8.25** ± **0.20**$^{a}$ | **6.81** ± 0.41$^{cd}$ | **6.45** ± 0.42$^{c}$ | **5.82** ± 0.45$^{cd}$ | **69.8** |
| **Bo 52** | **8.41** ± 0.32$^{a}$ | **7.98** ± 0.29$^{a}$ | **7.24** ± 0.22$^{a}$ | **6.85** ± 0.14$^{a}$ | **81.4** |
| **Bo 37** | **8.36** ± 0.44$^{a}$ | **8** ± 0.12$^{a}$ | **7.39** ± 0.1$^{a}$ | **6.57** ± 0.22$^{ab}$ | **78.5** |
| **Bo 3** | **8.39** ± 0.39$^{a}$ | **7.98** ± 0.33$^{a}$ | **6.58** ± 0.40$^{bc}$ | **6.35** ± 0.33$^{abc}$ | **75.6** |
| **RM 39** | **8.2** ± 0.16$^{a}$ | **7.36** ± 0.21$^{bc}$ | **6.55** ± 0.06$^{c}$ | **6.20** ± 0.65$^{bcd}$ | **75.6** |
| **RM 28** | **8.19** ± 0.28$^{a}$ | **7.51** ± 0.07$^{ab}$ | **7.29** ± 0.06$^{a}$ | **6.49** ± 0.40$^{ab}$ | **79.2** |
| **RM 3** | **8.51** ± 0.46$^{a}$ | **7.45** ± 0.54$^{ab}$ | **6.99** ± 0.26$^{ab}$ | **6.66** ± 0.17$^{abc}$ | **78.2** |

$^{abcde}$ Means in the same column followed by different superscript letters are significantly different ($P < 0.05$).
BO: – Boza & RM: – *Rayeb* Milk.
* Results are expressed as mean ± SD, each value is the average of three experiments and each was carried out in duplicate.

Fig. 1 illustrates the effect of two different phenol concentrations (0.2% and 0.5%) on the growth of nine LAB isolates after 24 h of incubation in MRS medium at 37 °C. The examined LAB isolates showed different degrees of sensitivity towards different concentrations of phenol. The highest tolerance (95.89%) to 0.2% phenol concentration was demonstrated for BO37 followed by RM39 (94.1%), while, BO52 had the lowest tolerance. At 0.5 phenol concentration, all the tested LAB isolates exhibited varying relative growth percentage ranged between 15.1% and 21.7%. Vizoso Pinto et al. (2006) observed varying degrees of sensitivity for four strains of *Lactobacillus johnsonii* and six strains of *L. plantarum* towards 0.4% phenol concentration while *L. plantarum* strains were less sensitive.

*Cholesterol removal by LAB isolates*

Hypercholesterolemia (elevated blood cholesterol level) is considered a major risk factor for the development of coronary heart disease. Therefore, lowering the serum cholesterol level is important to prevent the disease. The cholesterol – removing ability of LAB isolates was assessed *in vitro* in the presence of oxgall after 24 h of anaerobic growth at 37 °C (Fig. 2). All the nine LAB isolates showed the ability to remove cholesterol from the media. They exhibited varying degrees of cholesterol lowering ability ranged from 8.4% to 43.5%. The BO37 isolate manifested superior ability (43.75%) to remove cholesterol from the medium which was significantly higher than those of the other examined LAB isolates. The lowest value of cholesterol assimilation was traced in BO35 isolate. The ability of *in vitro* cholesterol level reduction in model culture media has been shown for numerous strains of LAB (Pereira and Gibson, 2002; Lavanya, 2001; Wang et al., 2012; Miremadi et al., 2014). Further studies are required to determine the mechanism(s) involved in the removal of cholesterol by those probiotic LAB isolates.

*Screening of LAB isolates for BSH activity*

*Qualitative determination of bile salts hydrolases activity*

The ability of probiotic strains to detoxify bile salt by producing BSH enzyme activity has often been included among the

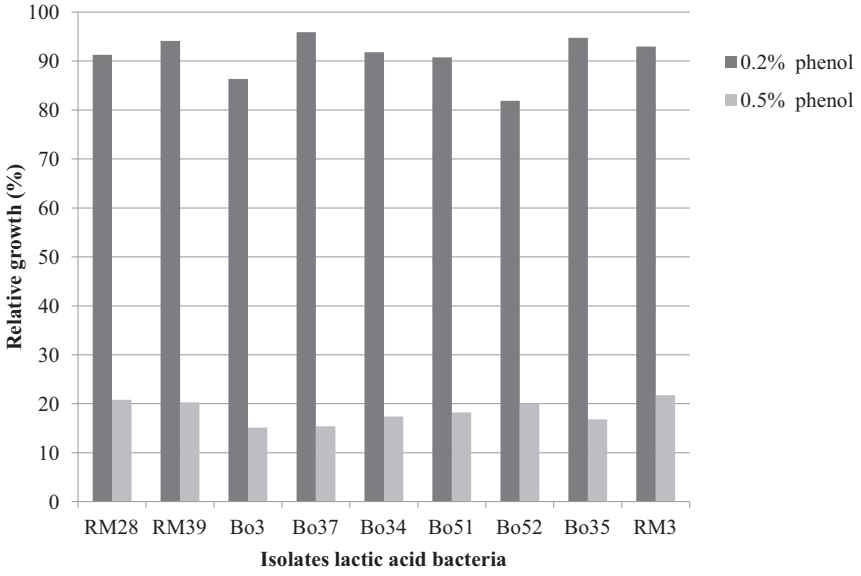


**Fig. 1** Effect of phenol concentration on the growth of LAB isolates. BO: – Boza & RM: – *Rayeb* Milk.

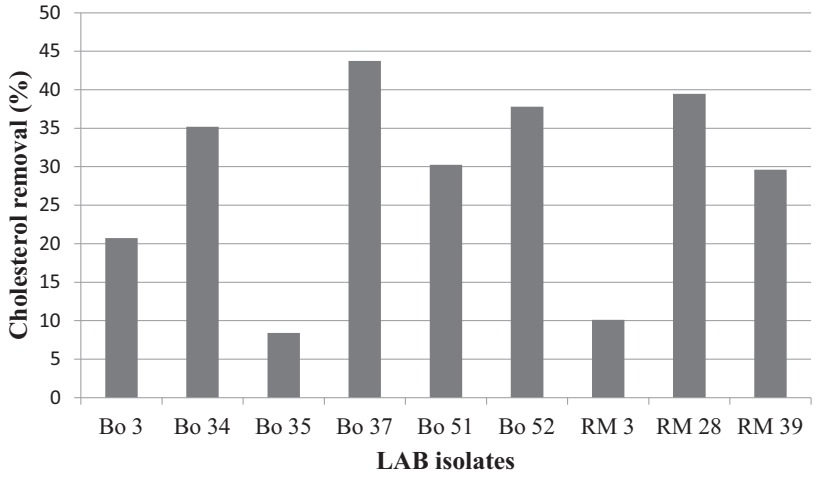


**Fig. 2** Cholesterol removal by LAB isolates. BO: – Boza & RM: – *Rayeb* Milk.

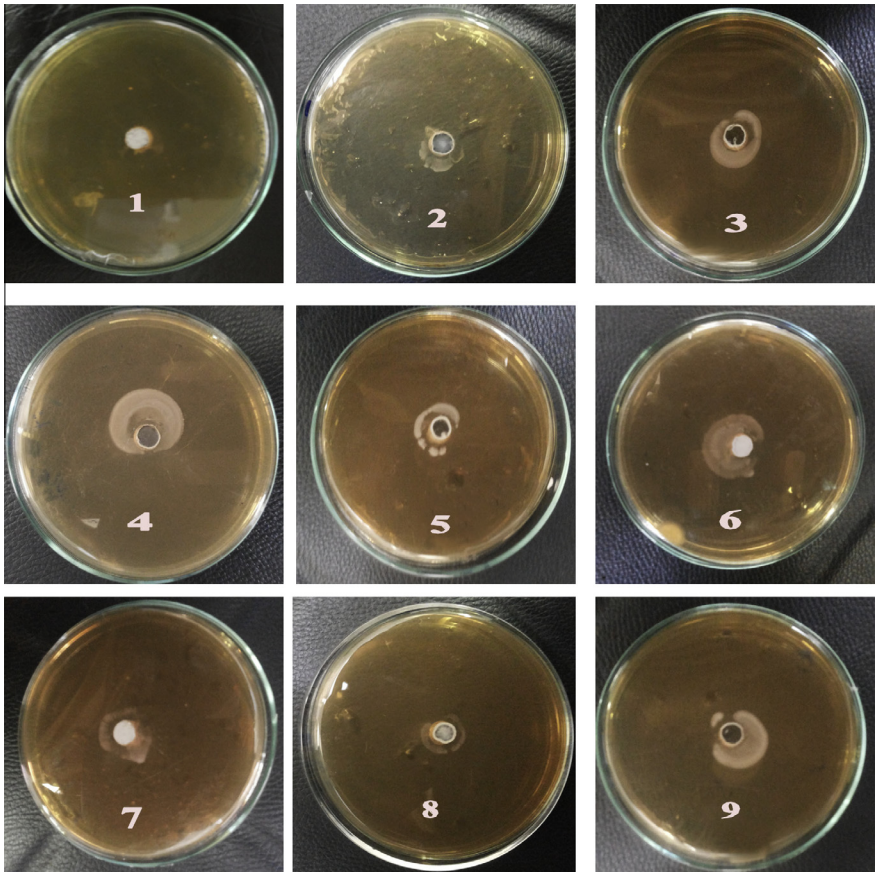


**Fig. 3** The BSH activity of LAB isolates grown on bile salt – MRS medium as manifested by the formation of precipitation zone around the colony. The code numbers 1–9 represent the LAB isolates as follows: 1: BO3; 2: BO34; 3: BO35; 4: BO37; 5: BO51; 6: BO52; 7: RM3; 8: RM28; 9: RM39. BO: – Boza & RM: – *Rayeb* Milk.

criteria for probiotic strain selection (Noriega et al., 2006). Bile salt hydrolase is an enzyme that catalyses the deconjugation of bile salt to liberate free primary bile acids (Gilliland and Speck, 1977).

When bile salt hydrolase producing LAB isolates were streaked on MRS plates containing TDCA, the taurine conjugated bile acid was deconjugated producing deoxycholic acid. The deconjugation activity of LAB isolates was manifested in Fig. 3, and copious amounts of deoxycholic acid precipitated around active colonies and diffused into the surrounding medium.

Out of nine LAB isolates previously selected based on their high antimicrobial activity, eight isolates displayed BSH activity to different levels. Four isolates (BO35, BO37, BO52,

RM39) exhibited high BSH activity by providing large precipitation zones (2.03, 2.45, 2.25, and 1.98 mm, respectively) around colonies on plate assay. Notwithstanding, the other four isolates demonstrated low BSH activity by expressing small (less than 1.5 mm) precipitation zones.

The presence of bile salt hydrolase (BSH) in probiotics renders them more tolerant to bile salts, which also helps to reduce the blood cholesterol level of the host (Noriega et al., 2006).

Contrary to the results of the present study, Begley et al. (2006) reported that BSH activity has not been detected in bacteria isolated from environments from which bile salts are absent. It is worthy to mention that all the eight BSH-positive LAB isolates are not associated with gastrointestinal environment.

*Quantitative determination of bile salts hydrolases activity*

The highest total BSH activity (3.09 u/ml) towards taurocholate was displayed by RM39 LAB isolate compared with other LAB isolates Table 6. In contrast, the lowest total BSH activity (0.25 u/ml) was demonstrated for RM3 isolate. Specific activity of BSH did not correlate well with total BSH activity by most LAB isolates due to varying protein content in cell extracts. The RM39 and RM3 isolates had high (3.09 u/ml) and low (0.25 u/ml) total BSH activity, respectively and exhibited the same trend as well with respect to the specific activity. Meanwhile, BO37 isolate that had high total BSH activity (2.47 u/ml) displayed low specific activity (0.85 u/mg). Similar results were reported by Liong and Shah (2005) for lactobacillus strains towards different bile salts. Several studies have indicated that the mechanism for *in vitro* removal of cholesterol is linked to the bile salt hydrolase activity of probiotic strains (Kimoto et al., 2002; Liong and Shah, 2005). Moreover, the decomposition of bile salts by BSH enzyme would disrupt the formation of the cholesterol micelle which in turn prevents cholesterol absorption (Klaver and Van der Meer, 1993).

From the genetic data, it was obvious that BSH is an intracellular enzyme. This is consistent with the observation that no enzyme activity was present in the supernatants of overnight cultures, while activity was released either by sonication or other cell disruption methods or by lysis in assays performed with whole cells due to the lytic properties of the bile salts (Lunden and Savage, 1990; Grill et al., 1995; Tanaka et al., 2000).

**Table 6** BSH activity of lactic acid bacteria isolates on sodium taurocholate.[a]

| Isolates | BSH activity[b,c] | | |
|---|---|---|---|
| | Total protein (mg/ml) | Total activity (U/ml) | Specific activity (U/mg) |
| Bo 34 | 1.22 ± 0.27$^{b}$ | 1.64 ± 0.28$^{d}$ | 1.34 ± 0.32$^{a}$ |
| Bo 35 | 2.62 ± 0.61$^{a}$ | 2.31 ± 0.22$^{bc}$ | 0.88 ± 0.25$^{cd}$ |
| Bo 37 | 2.89 ± 0.26$^{a}$ | 2.47 ± 0.45$^{b}$ | 0.85 ± 0.09$^{d}$ |
| Bo 51 | 1.08 ± 0.44$^{b}$ | 0.67 ± 0.34$^{e}$ | 0.62 ± 0.5$^{e}$ |
| Bo 52 | 2.14 ± 0.89$^{a}$ | 1.95 ± 0.15$^{cd}$ | 0.91 ± 0.52$^{c}$ |
| RM 3 | 0.42 ± 0.49$^{b}$ | 0.25 ± 0.15$^{ef}$ | 0.59 ± 1.19$^{e}$ |
| RM 28 | 2.54 ± 0.66$^{a}$ | 2.25 ± 0.24$^{bc}$ | 0.88 ± 0.16$^{cd}$ |
| RM 39 | 2.96 ± 0.11$^{a}$ | 3.09 ± 0.27$^{a}$ | 1.04 ± 0.13$^{b}$ |

BO: – Boza & RM: – *Rayeb* Milk.

[a] Results are expressed as means ± standard deviation; values are means of triplicate.

[b] BSH activity from cell free extracts of lactic acid bacteria isolates grown on MRS broth supplemented with 6 mM sodium taurocholate.

[c] Means in the same column followed by different superscript letters are significantly different ($P < 0.05$).

*Identification of promising LAB isolates*

*Phenotypic characterization*

The seven promising isolated strains (BO3, BO34, BO37, BO51, BO52, RM28, RM39) are Gram positive, catalase negative, non-spore forming and fermenting glucose. All the isolates are rod shaped except RM39 and BO37 that are spherical cells. The physiological and biochemical characteristics of the selected LAB isolates Table 7 are similar to those described in Bergey's Manual of Determinative of Bacteriology (Logan and De Vos, 2009) for the genera *Lactobacillus* and *Lactococcus*. Further, biochemical characterization using API 50 CHL for *Lactobacilli* and API 20 for cocci showed the similarity in characteristics with the corresponding identified LAB species (Table 8). Bill et al. (1992) and Klinger et al. (1992) indicated that some commercial identification systems often yield good results regarding genus identification but they were not fully adequate at the species level.

**Table 7** Physiological characteristics of lactic acid bacteria isolates.

| Characteristics | LAB isolates | | | | | | |
|---|---|---|---|---|---|---|---|
| | BO 3 | BO 34 | BO 37 | BO 51 | BO 52 | RM 28 | RM 39 |
| Gram strain | + | + | + | + | + | + | + |
| Catalase production | – | – | – | – | – | – | – |
| Glucose fermentation | – | – | – | – | – | – | – |
| *Growth:* | | | | | | | |
| 15 °C | + | – | + | + | + | – | + |
| 37 °C | + | + | + | + | + | + | + |
| 45 °C | + | + | – | + | + | + | – |
| *NaCl* | | | | | | | |
| 2% | + | + | + | + | + | + | + |
| 4% | + | + | + | + | + | + | + |
| 6.5% | – | – | + | + | + | – | + |

BO: – Boza & RM: – *Rayeb* Milk.

**Table 8** Identification of LAB isolates by API 50 CHL and API 20 kits.

| Isolates | Species identified by API test |
|---|---|
| BO 3 | *Lactobacillus rhamnosus* |
| BO 34 | *Lactobacillus delbrueckii* subsp. *bulgaricus* |
| BO 37 | *Lactococcus lactic* subsp. *lactis* |
| BO 51 | *Lactobacillus paracasei* |
| BO 52 | *Lactobacillus paracasei* |
| RM 28 | *Lactobacillus gasseri* |
| RM 39 | *Lactococcus lactic* subsp. *lactis* |

BO: – Boza & RM: – *Rayeb* Milk.

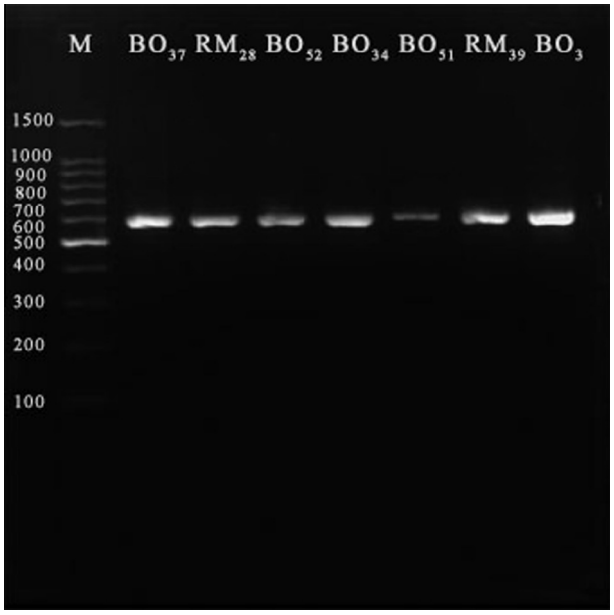


**Fig. 4** PCR products for lactic acid bacteria isolates (BO37; RM28; BO52; BO34; BO51; RM39 and BO3) in a 2% agarose gel. Lane 1 (DNA Ladder from 100 bp to 1500 bp), lanes 2–7 (isolates lactic acid bacteria). BO: – Boza & RM: – *Rayeb* Milk.

*Molecular identification*

Molecular methods are important for bacterial identification (Drancourt et al., 2000; Sghir et al., 2000; Greetham et al., 2002; Heilig et al., 2002) and possibly more accurate for LAB than the conventional phenotypic methods. In the present study, 16sr-DNA of the total genomic DNA from promising LAB isolates was amplified and sequenced for identification. Amplification using universal primer produced a PCR product of approximately 600 bp (Fig 4). The sequencing data of purified 16S r-DNA of isolates were employed for bacterial identification. The sequences of the selected isolates were aligned with the 16S r-DNA sequences from the GenBank database (website) to identify the studied microorganism. 16S r-DNA sequencing data of the selected isolates clearly showed (BO 3) 90% homology to *Lactobacillus rhamnosus* & (BO 34) 96% homology to *Lactobacillus delbrueckii* subsp. *Bulgaricus* & (BO 37) 99% homology to *Lactococcus lactic* subsp. *lactis* & (BO 51) 100% homology to *Lactobacillus paracasei* & (BO 52) 100% homology to *Lactobacillus paracasei* & (RM 28) 100% homology to *Lactobacillus gasseri* and (RM 39) 98% homology to *Lactococcus lactic* subsp. *lactis*.

## Conclusion

In the present study, 142 LAB strains were isolated from different sources and only nine isolates were selected on the basis of their high antagonistic activity. The nine promising LAB isolates exhibited good resistance to gastrointestinal conditions (pH, 2; bile salt, 0.3%; phenol 0.2–0.5%), high cholesterol removal and expressed BSH activity. Among the promising LAB isolates, BO37 which isolated from *Boza* and identified as *Lactococcus lactic* subsp. *lactis* manifested the highest cholesterol removal ability (43.7%) and good BSH activity (2.47 u/ml). Accordingly, owing to its good probiotic properties, this strain could be potentially used in functional food and health products especially where cholesterol reduction in food is the main target. Further *in vivo* study is necessary to prove the hypocholesterolemic effect of the isolated *Lactococcus lactic* subsp. *lactis*. Moreover *in vitro* studies are required to determine the mechanism(s) involved in the reduction of cholesterol by such a promising isolate.

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