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First Isolation and Molecular Detection of Autochthonous Potential Probiotic Lactobacilli Isolates from Iranian Traditional Poosti Cheese and their Antioxidative Activity

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Abstract

Introduction: Poosti cheese is an artisanal cheese produced mainly from ewe's milk in southwest Iran. The objective of this study was to identify lactobacilli isolated from Poosti cheese and to investigate their potential probiotic and antioxidative properties.

Materials and methods 101 Gram-positive bacilli with catalase-negative reaction were isolated from Poosti cheese. Twenty isolates were selected based on their highest acid and bile salt tolerability. Using genus-specific primers, 10 isolates out of these were identified as *Lactobacillus*, and they were subsequently identified at species level using 16S rRNA gene sequencing. *In vitro* tests were used to assess their probiotic properties or antioxidative activities.

Results: Six species were *Lactobacillus plantarum*, and the rest was *Lactobacillus paracasei*, *Lactobacillus acidophilus*, *Lactobacillus brevis*, and *Lactobacillus buchneri*. The acid and bile tolerances were quantitatively close to the reference probiotic strain. *L. plantarum* S32E showed the highest survival under simulated gastric or intestinal conditions. Cholesterol reduction ability of *L. plantarum* S12E and *L. acidophilus* S37A was significantly higher than that of the reference strain. *L. acidophilus* S37A and *L. plantarum* S12E were more susceptible against common antibiotics. It was found that the highest antimicrobial activity was detected in the case of *Listeria monocytogenes*, and the extent of the activity was species- and strain-dependent. The best result of DPPH radical scavenging activity and hydroxyl radical scavenging activity was achieved by intact cells of *L. paracasei* S5D and by intracellular cell-free extracts of *L. plantarum* S8D, respectively. The results indicated that the superoxide dismutase activity of *Lactobacillus* could not be regarded as an appropriate predictor of antioxidative traits. The highest glutathione content belonged to *L. acidophilus* S37A and *L. parcasei* S5D.

Discussion and conclusion: Overall, some of *Lactobacillus* strains of Poosti cheese could be considered as potential probiotic candidates; it should, however, be confirmed by further *in vivo* evaluation.

Key words: Antioxidative Activity, Lactobacilli, Poosti Cheese, Probiotic

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Introduction

Traditional dairy fermented foods are one of the most well-known and bountiful products that have ever existed in human history. These foods can contain an important category of useful and non-pathogenic microorganisms that are classified as probiotics. Regardless of their origins, when ingested in sufficient numbers, probiotics confer health benefits on the consumer (1, 2). Among different genera of microorganisms which have been proposed as probiotics, *Lactobacillus* and *Bifidobacterium* are the most important ones (3). Lactobacilli are members of lactic acid bacteria (LAB) and contain over 100 species (4). Research findings have shown that many of species of starter or nonstarter lactobacilli such as *L. plantarum*, *L. casei*, *L. delbrueckii*, *L. rhamnosus*, *L. brevis*, *L. acidophilus*, *L. fermentum,* and *L. helveticus* are characterized to ferment carbohydrate and to extend the shelf-life of food products (5). Beside other genera of probiotics, *Lactobacillus* spp. could result in the production of antimicrobial substances, prevention of gastrointestinal colonization by pathogenic microorganisms, modulation of the function of the human immune system, reducing blood cholesterol, reducing the risk of cancers and so forth (3, 6).

A variety of potent oxidants including reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical, hydrogen peroxide and singlet oxygen is permanently generated in the body of living organisms, playing a major role in the pathogenesis of different diseases or disorders such as rheumatoid arthritis, amyloidosis, Alzheimer’s disease, atherosclerosis, carcinogenesis and mutagenesis (7, 8). Both synthetic and natural antioxidants have been used successfully to reduce ROS-triggered oxidative damage. However, synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole and tertiary butylhydroquinone may contribute to tumorigenicity and carcinogenicity (9). Therefore, the focus of numerous researches has been drawn to the natural nutritive or nonnutritive antioxidants. Some lactobacilli are reported to have the potency to decrease the induced oxidation by ROS (6, 10, 11). As a result, in addition to a supplemented typical LAB in functional foods, wild isolated LAB from artisanal dairy products could be considered as potential candidates due to their potential biological properties.

Traditional fermented foods have been consumed for centuries by Iranian people. The Poosti cheese as a dairy fermented product has been traditionally produced since the ancient times form raw ewe’s and goat’s milk alone or mixed by Qashqai and Bakhtiari nomadic tribes in southwest Iran. Traditionally, the milk curd is obtained by adding powdered rennet extracted from abomasum of young milk-fed lambs or kids in which the important active ingredient is chymosin. In addition, rennet alternatives derived from other sources are now used by the producer tribes. Consequently, whey separation is carried out by putting the product in cloth bags. Finally, the coagulant is introduced into the tanned skin of sheep or goat. This is followed by air removal, knotting and closing the skin in which the curd is left to rest at least one month until it has ripened.

To the best of our knowledge, no attention has been paid to the investigation of the different chemical or microbial properties of the Poosti cheese in authentic literature so far. Therefore, the present study is the first investigation on isolation, molecular characterization and antioxidative activity evaluation of wild lactobacilli in artisanal Poosti cheese ever reported.

Materials and Methods

Sampling and isolation of lactobacilli: The Poosti cheese samples produced in Chaharamahal and Bakhtiari and Kohgiluye and Boyerahmad provinces, located in southwest Iran, were collected from April to July 2016. Distinctly, every six days for one hundred and twenty days, twenty batches with three samples were obtained from geographically dispersed nomadic tribes. The samples were put in sterile polyethylene zipper bags and immediately transported to the laboratory and stored at 4 °C until analysis.

A 10-g portion of each cheese sample was placed in a filtered sterile stomacher bag and diluted and homogenized with 90 ml of buffered peptone water for 2 min using a stomacher (Seward 400, UK). Serial dilutions of the homogenate were obtained by adding the sample to the same diluent. A100-µl portion of each dilution was plated onto de Man, Rogosa and Sharp (MRS) agar and incubated at 37 °C for 72 h. The selectivity of the culture was previously increased by the addition of 100 µg/ml of cycloheximide (2). Thereafter, colonies showing different morphologies were selected and purified by successive streaking on lactobacilli MRS agar. In all the experiments, *L. rhamnosus* ATCC 7469 was used as a positive reference probiotic strain.

Primary screening of isolates: The isolates were preliminarily identified according to their Gram staining, cell morphology and catalase activity. As a result, isolates of Gram-positive bacilli with catalase-negative reaction were confirmed and selected for further testing. The isolates were subcultured and kept at –80 °C in Lactobacilli MRS broth supplemented with 20% (v/v) sterile glycerol as a cryoprotective agent.

***In vitro* screening for the potential probiotic characteristic of isolates**

Acid resistance of isolates: The acid resistance of isolates was screened according to Lee et al. (12), with slight modifications. The isolates were incubated at 37 °C in MRS broth till OD_{600} nm reached 0.8. 0.5 ml of centrifuged (10000 × *g*, for 10 min) and PBS-washed bacterial cells were transferred and re-suspended to 4.5 ml MRS broth which its pH was previously adjusted at 2 or 3 with 1 M HCl. The cultures were incubated at 37 °C and the OD_{600} nm was recorded every 60 min to 3 h as the routine transit time of food through the human stomach. MRS broth without pH adjustments was also incubated in the same way as the control. All the measurements were repeated three times. The acid resistance was calculated as follows:

$$\text{Survival (\%)} = \frac{\text{OD600 of MRS broth at pH 2 or 3}}{\text{OD600 control}} \times 100$$

Bile salts tolerance of isolates: The tolerance to bile salts of isolates was evaluated according to Lee et al. (12) and Walker and Gilliland (13) with some modifications. In brief, isolates were inoculated to MRS broth, and incubated at 37 °C until the OD_{600} nm reached 0.8. 0.5 ml of centrifuged (10000 × *g*, for 10 min) and PBS-washed bacterial cells were transferred and re-suspended to 4.5 ml MRS broth containing 0.15 and 0.3% (w/v) bile salts (Sigma-Aldrich). The cultures were incubated at 37 °C and the OD_{600} nm was measured every 80 min for 4 h reflecting the time spent by food in the human small intestine. MRS broth without bile salts was also prepared in the same way as the control. The growth rate of test culture was compared to the control culture. All the measurements were repeated three times. The survival rate was measured as follows:

$$\text{Survival (\%)} = \frac{\text{OD600 (MRS +bile salts)}}{\text{OD600 control}} \times 100$$

Molecular characterization of isolates: Out of the 101 isolates, 10 were selected for molecular characterization on the basis of their strong acid and bile salts tolerance properties. Genomic DNA was extracted with a DNA extraction kit for Gram–positive bacteria (MBST, Tehran, Iran), according to manufacturer's instructions, and stored at –20 °C. The amount of extracted DNA was determined using a UV-visible spectrophotometer at 260 and 280 nm, and the purity was checked by 1% agarose gel electrophoresis. Chromosomal DNA was used as a template for PCR reactions. The primer pairs (Forward 5′-TGGAAACAGGTGCTAATACCG-3′ and reverse 5′-GTCCATTGTGGAAGATTCCC-3′), and (Forward 5′-AGAGTTTGATCCTGGCTCAG-3′ and (reverse 5′-ACGGCTACCTTGTTACGAC-3′) applied for the amplification of 16s rRNA gene, were designed using the primer3 software from the library of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), and synthesized by Takapouzist Co (Tehran, Iran). The PCR mixture comprised of 2.5 µl PCR buffer, 0.5 µl dNTP, 1.5 µl $MgCl_2$, 0.2 µl Taq DNA polymerase, 1 µl each of forward and reverse primers, and 500 ng of template in a final volume of 25 µl. The amplification was performed in an automatic Thermal Cycler (MJ Mini, Bio-Rad) using the following conditions: 1 min of initial denaturation at 95 °C, denaturation for 40 s at 94 °C, primer annealing for 30 s at 56.5 °C, primer elongation for 30 or 90 s at 72 °C, repeat (30 cycles) with a final elongation for 10 min at 70 °C. In all PCR reactions, instead of DNA template, Milli–Q water was applied as a negative control. PCR products were subjected to gel electrophoresis in 1.1 % (w/v) agarose. Thereafter, the agarose gel was stained with DNA Safe Stain (Sinaclone, Iran), visualized on a UV illumination box, and photographed using a digital camera.

In addition, species identification was done by 16srRNA gene sequencing. For this purpose, the resulting PCR products were purified using the AccuPrep® PCR Purification Kit (Takapouzist Co., Tehran, Iran), and sequenced with both forward and reverse primers on an ABI 3730XL DNA Analyzer (Bioneer, Daejeon, South Korea). The obtained sequences were compared to related sequences using basic local alignment search tool (www.ncbi.nlm.nih.gov/blast) and were deposited in the GenBank database to receive accession numbers.

Tolerance of identified isolates to simulated gastric and intestinal conditions: The *in vitro* tolerance of identified lactobacilli to simulated gastric or intestinal environment was evaluated, as described previously, with modifications (14). Briefly, the overnight cultured bacteria in MRS broth were centrifuged, washed and re-suspended in PBS to achieve an approximate cell density of 10^8 CFU/ml. One ml of the dilutions was exposed to 9 ml of simulated gastric juices (3 g/l pepsin; 125 mM NaCl; 17 mM KCl; 45 Mm NaHCO3) with pH 2.5 as the average pH in stomach of human, and was incubated at 37 °C in shaker incubator at 150 rpm for 180 min. Free-simulated gastric cultures were incubated in the same way as the control. Thereafter, pellets of the survived bacteria were harvested by centrifuging and re-suspended in PBS to a density of approximately 10^8 CFU/ml. One ml of the cell suspensions was added to 9 ml of simulated intestinal juice (1 mg/ml pancreatin; 1.5 mg/ml ox bile salts) with pH 8 as the average pH in the small intestine of human and incubated as described previously. Free-simulated intestinal cultures were incubated in the same way as the control. The percentage of survival was measured as follows:

$$\text{Survival (\%)} = \frac{\text{Log CFU/ml after 3 h incubation in gastric or intest}}{\text{Log CFU/ml after 3 h incubation in free-gastric or int}} \times 100$$

Assays were carried out in triplicate.

Cholesterol assimilation ability: Ten mg of water–soluble cholesterol (Sigma-Aldrich) was added to 1 ml of Milli–Q water and filtered through a 0.22 µm membrane. Fifty µl of the obtained solution was transferred to 5 ml of MRS broth supplemented with 0.3 % w/v oxgall to achieve a final cholesterol concentration of 100 µg/ml. The overnight activated culture of each of the 10 *Lactobacillus* strains was inoculated into the MRS broth and incubated anaerobically at 37 °C for 24 h. Un–inoculated MRS broth was also used as a control. Thereafter, bacterial cells were removed by centrifugation (12000 × *g*, for 10 min), and the supernatant was used to determine the residual cholesterol concentration using a modified method as described by Rudel and Morris (15). 0.5 ml of the supernatant was mixed with 3 ml of absolute ethanol and 2 ml of KOH (50%, w/v). The mixture was heated at 60 °C for 10 min and then allowed to cool to 25 °C. 5 ml of hexane was carefully added and vigorously vortexed for 30 s. Then, 3 ml of Milli–Q water was added, and the mixture was maintained for 15 min at ambient temperature for the separation of the organic phase and aqueous phase. 2.5 ml of the upper hexane–included phase was transferred into a glass tube and the hexane was evaporated under pressure of 10 psi at 60 °C. The residue was re-suspended in 4 ml of Ophthalaldehyde reagent, mixed and kept for 10 min. 2 ml of concentrated sulfuric acid was gradually added to the tube, and the reaction solution was allowed to stand for 10 min. The absorbance values were recorded at 550 nm.

Antimicrobial activity and antibiotic susceptibility: A conventional agar well diffusion assay with minor modifications was used in triplicate to assess the antimicrobial activity of the 10 *Lactobacillus* strains against *Listeria monocytogenes* ATCC 19118, *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 14028, and *Escherichia coli* O157:H7 43895 (1, 16). Overnight incubated *Lactobacillus* strains in MRS broth were centrifuged (10000 × 10 g, for 10 min), and the relevant supernatants were recovered. The inoculums were prepared from 24 h TSB broth cultures of each target strain containing approximately 10^6 CFU/ml and were uniformly spread over Muller Hinton agar. Wells of 6 mm in diameter was then punched in the inoculated plates and loaded with filter–sterilized supernatants of each *Lactobacillus* strain. The plates were incubated at 37 °C for 24h and were then assessed for the zone of inhibitions (ZOI).

Antibiotic susceptibility of the 14 *Lactobacillus* strains was done using disc diffusion method and interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (17). The following antibiotic–containing discs were used: penicillin (10 µg), ampicillin (10 µg), cefixime (5 µg), gentamicin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), Ciprofloxacin (5 µg), and clindamycin (2 µg). Fresh overnight cultures were diluted to a final dose of 10^6 to 10^7 CFU/ml, and subsequently inoculated on MRS agar to achieve a confluent layer. The discs were placed on the agar surface. After incubation for 24 h at 37 °C, the ZOIs (mm) were measured with a digital caliper. At least three replicates per *Lactobacillus* strain were carried out.

Evaluation of antioxidative activity

Preparation of bacterial cells and extracts: The overnight cultured cells of the 10 *Lactobacillus* strains were harvested by centrifugation at 3500 × 10 g, for 15 min at 4 °C and then washed twice with phosphate-buffered saline (PBS, pH 7.2). The washed pellets were re-suspended in sterile double deionized water and total cells counts were adjusted to about 10^9 CFU/ml. Thereafter, the intracellular cell-free extracts (mainly cytoplasmic extracts) were prepared by

ultrasonic disruption (pipe Lab750, Sinaptech, France) of bacterial cells. The ultrasonication was done for five 60 s strokes in an ice bath, with 5-min intervals. The cell debris was removed by centrifugation (10000 × 10 g, for 15 min). Finally, the intact cells or cleared intracellular cell-free extracts were used to assess the antioxidative activity.

Scavenging of DPPH radicals: The DPPH radical-scavenging potency of the 10 *Lactobacillus* strains was evaluated according to the method of Lin and Chang (18). In brief, 200 µl of intracellular cell-free extract or intact cells solution (10^9 CFU/ml) was added to 250 µl of a freshly prepared ethanolic DPPH solution of (0.2 mM/l), mixed vigorously and left to stand for 30 min in the dark at ambient temperature. The double deionized water and DPPH solution was considered as the control. The absorbance was read at 517 nm and the DPPH scavenging activity was calculated in the following way:

$$\text{Scavenging activity (\%)} = \left(1-\left(\frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right)\right) \times 100$$

Scavenging of hydroxyl radicals: The hydroxyl radical scavenging activity was evaluated using a Fenton reaction method (19) with a slight modification. In this reaction, hydroxyl radicals (OH) are generated via H_2O_2 decomposition by Fe^{2+}. The hydroxyl radicals can then attack the central carbon atom of a cationic dye such as brilliant green. Hydroxyl radical scavengers can prevent oxidation degradation of brilliant green in Fenton reaction (20). In brief,1.0 ml of brilliant green (0.435 mM), 2.0 ml of ferrous sulfate (0.5 mM), 1.5 ml hydrogen peroxide (3.0 % w/v), and different volumes (1, 1.5, 2 and 2.5 ml) of intracellular cell-free extract or intact cells solution (10^9 CFU/ml) were added consecutively in a tube and incubated at ambient temperature for 25 min. The absorbance was immediately measured at 624 nm and the hydroxyl radical scavenging activity was calculated using the following equation:

$$\text{Scavenging activity (\%)} = \left[\frac{(As-Ac)}{(A0-Ac)}\right] \times 100$$

Where A_S and A_C are the absorbance of the sample (in the presence of sample) and the control (in the absence of sample), respectively. A_0 is the absorbance without both the sample and Fenton reaction system.

Superoxide dismutase activity of the intracellular cell-free extract: The superoxide dismutase (SOD) activity was assayed using a xanthine oxidase/nitroblue tetrazolium salt (XO/NBT) system as described previously (21). In this system, the reaction between xanthine and xanthine oxidase in the presence of NBT salt can form a red formazan dye. Inhibition of this reaction by a given sample represents its SOD activity. In brief, 2.4 ml of sodium carbonate buffer (50 mM; pH 10.2), 0.1 ml of xanthine (3 mM), 0.1 ml of ethylenediaminetetraacetic acid (3 mM), 0.1 ml of NBT (0.75 mM), 0.1ml of bovine serum albumin (15%) and 0.1 ml of cell-free extract or 0.1 ml of double deionized water (as control) were added together. 0.1 ml of xanthine oxidase was added to the reaction mixture whose absorbance changes at 560 nm were recorded at 25 °C for 20 min. The SOD activity was measured as U/mg.

Glutathione content of the intracellular cell-free extract: Total glutathione (TGSH) and glutathione (GSH) of cell lysates were assayed using a commercially available kit (ZellBio GmbH, Germany) according to the manufacturer's instructions. In this assay, the reaction between glutathione and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) generates 2-nitro 5-thiobenzoic acid (TNB) which has a yellow color. The TNB was detected by the kit and glutathione content was determined by measuring absorbance at 412 nm. The oxidized glutathione (GSSG) concentration was calculated by subtraction the TGSH content from the GSH content.

Statistical analysis: The data analysis was carried out using the GraphPad InStat 3 statistical software package (San Diego, CA). One-way ANOVA followed by Tukey's multiple comparison test or *t*-test was used for the statistical comparisons of the data. *P* values less than 0.05 were considered significant.

Results and discussions

Isolation and primary screening and molecular characterization: Of total 147 isolated strains, 101 isolates were Gram-positive, rod-shaped and catalase-negative bacteria which were selected for further testing. According to our strategy, 20% of isolates were selected followed by a preliminary screening of probiotic potentiality based on their best ability to survive at low pH and in the presence of bile salts. PCR amplification using genus-specific primers revealed that 10 isolates were lactobacilli. The lack of appropriate accuracy and precision of biochemical tests compared to molecular methods in identifying lactobacilli at species level has been previously reported (22). Therefore, further partial sequencing of 16s rRNA gene was applied to characterize lactobacilli at species level as shown in Table 1. The sequence homology of the identified strains ranged from 98% to 100%. The dominant *Lactobacillus* species detected in Poosti cheese isolates were *L. plantarum* (12E, S7B, S14D, S8D, S32E, and S8C) and the rest were *L. paracasei* (S5D), *L. acidophilus* (S37A), *L. brevis* (S15B), and *L. buchneri* (S15C). Similarly, the highest proportion of Lactobacilli which were isolated from Siahmazgi cheese as a similar Iranian fermented artisanal dairy product belonged to *L. planatrum* species (23).

***In vitro* screening for potential probiotic characteristics**

Acid resistance and bile salt tolerance of isolates: As shown in Table 1, all ten strains exhibited satisfactory resistance to low pH values of 2.0 and 3.0. Compared to the reference probiotic strain, three strains (S37A, S8D, and S5D), and five strains (S37A, S15C, S8D, S5D, and S8C) were survived more vigorously at pH values of 2.0 and 3.0, respectively. Beside the bile salt tolerance, the acid resistance is an important prerequisite for the probiotic potentiality of lactic acid bacteria (12).

Tolerance of isolates to bile salt concentrations is presented in Table 1. All of the tested strains were found to be resistant to 0.15 or 0.3% of bile salt concentrations. Four strains (S32E, S8D, S37A, and S15C) demonstrated higher survival ratios (%) than the reference probiotic strain (*L. rhamnosus* ATCC7469). The highest tolerance based on survival ratio% (78.33 ± 11.86%) was recorded for *L. planatrum* S32E. Bile resistance of lactobacilli could reflect their ability to survive in gastrointestinal transit, which enabled us to focus them as potential probiotic candidates (1).

Tolerance to simulated gastric and intestinal conditions: All identified lactobacilli strains maintained their survivability after exposure to simulated gastric juice. However, the prominent viability of some strains like *L. acidophilus* S37A, *L. plantarum* S32E, *L. buchneri* S15B, and *L. paracasei* S5D was absolutely comparable ($P>0.05$) to that from the reference probiotic strain whose survival ratio was equal to 89.89 ± 2.81% (Figure 1). Regarding tolerance to simulated intestinal juice, the survival ratio (%) of five strains (S32E, S8C, S5D, S37A, and S15B) was higher than 50% (Figure1). These findings are in agreement with other investigations which demonstrated the resistance to gastrointestinal conditions was strain-dependent (24, 25). Among the strains, *L. plantarum* S32E exhibited the

highest tolerance to simulated intestinal juice and even better than the reference probiotic strain.

Table 1- Identification of *Lactobacillus* isolates from Poosti cheese by 16s rRNA gene sequencing and their acid and bile salt tolerance.

Strain	The most matched species	GeneBank accession No. (16s rRNA)	Acid resistance (survival ratio, %)			Bile salts tolerance (survival ratio, %)		
			pH 2.0	pH 3.0	pH 2.0 and 3.0	0.15% bile salts	0.3% bile salts	0.2% and 0.3% bile salts
S12E	*L. plantarum*	MH400161	46.4 ± 2.71	56.98 ± 2.53*	51.69 ± 6.25	62.18 ± 1.95*	47.79 ± 2.07*	54.99 ± 8.08
S7B	*L. plantarum*	MH400154	38.74 ± 1.85*	66.03 ± 6.08	53.05 ± 16.58	61.78 ± 4.13*	30.16 ± 3.58*	45.97 ± 17.66
S14D	*L. plantarum*	MH400155	40.21 ± 0.95	60.45 ± 4.24*	48.15 ± 13.99	71.49 ± 5.54	42.46 ± 2.06*	56.98 ± 16.34
S8D	*L. plantarum*	MH400156	55.02 ± 3.02	84.24 ± 2.69*	69.63 ± 16.21	81.84 ± 1.11	59.75 ± 2.14	75.41 ± 7.22
S32E	*L. plantarum*	MH400157	38.49 ± 2.44	73.70 ± 2.75	56.10 ± 19.42	89.02 ± 2.34*	67.64 ± 1.86	78.33 ± 11.86
S8C	*L. plantarum*	MH400153	38.71 ± 2.06*	75.60 ± 2.69	57.16 ± 20.31	71.83 ± 2.86	46.14 ± 2.65*	58.99 ± 14.28
S5D	*L. paracasei*	MH400162	52.6 ± 2.37	80.78 ± 2.87	66.69 ± 15.61	71.12 ± 5.54	33.41 ± 2.60*	52.26 ± 21.02
S37A	*L. acidophilus*	MH400163	55.47 ± 2.04*	92.61 ± 3.22*	74.04 ± 20.48	86.39 ± 0.87*	59.75 ± 2.13	73.07 ± 14.66
S15B	*L. brevis*	MH236166	46.67 ± 3.23	64.10 ± 2.74	55.38 ± 9.92	50.37 ± 1.75*	36.28 ± 1.81*	43.32 ± 7.88
S15C	*L. buchneri*	MH400152	44.81 ± 3.20	88.28 ± 2.65*	66.04 ± 23.41	82.5 ± 1.54	55.20 ± 3.01	68.85 ± 15.11
ATCC 7469	*L. rhamnosus*	–	47.71 ± 2.69	72.59 ± 3.08	60.31 ± 13.95	74.64 ± 4.43	60.14 ± 3.79	67.92 ± 7.99

* Indicates significant difference ($P< 0.05$) with respect to the positive control (*L. rhamnosus* ATCC 7469 as the reference probiotic).

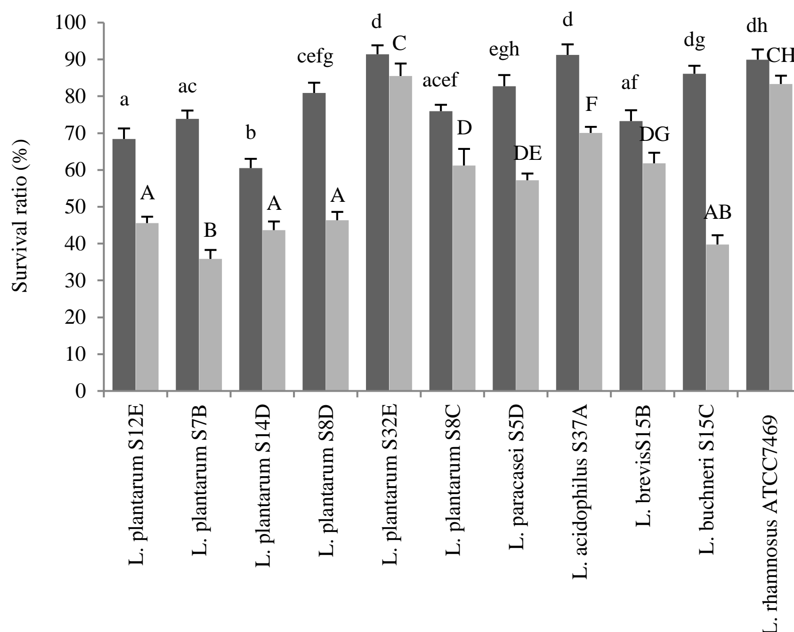

Fig. 1- Survival rates of *Lactobacillus* strains after exposure to simulated gastric juice and simulated intestinal juice. Different lower case letters (a-h) indicate significantly difference ($P<0.05$) between strains against simulated gastric juice. Different upper case letters (A-H) indicate significantly difference ($P<0.05$) between strains against simulated intestinal juice.

Cholesterol-lowering ability: It has been suggested that the cholesterol removal by certain lactobacilli is caused by the drop in the pH of the medium due to acid production parallel to microbial growth, which can result in disruption of cholesterol micelles followed by deconjugation and precipitation of cholesterol with the bile salts (26). Cholesterol adherence to bacterial cell walls and bile salt hydrolase activity may also have been involved in the cholesterol assimilation ability of lactobacilli (27). All the strains assayed were capable of assimilating of cholesterol from MRS broth. The quantity of cholesterol assimilated ranged from 26.76 ± 2.34 µg/ml to 53.23 ± 1.88 µg/ml (Figure 2). Notably, *L. acidophilus* S37 and *L. plantarum* S12E were significantly more efficacious (P<0.05) in cholesterol assimilation than the reference probiotic and other strains.

In vitro, screening of cholesterol-assimilating properties of lactobacilli strains is considered as an important criterion in their selection for complementary *in vivo* investigations (28).

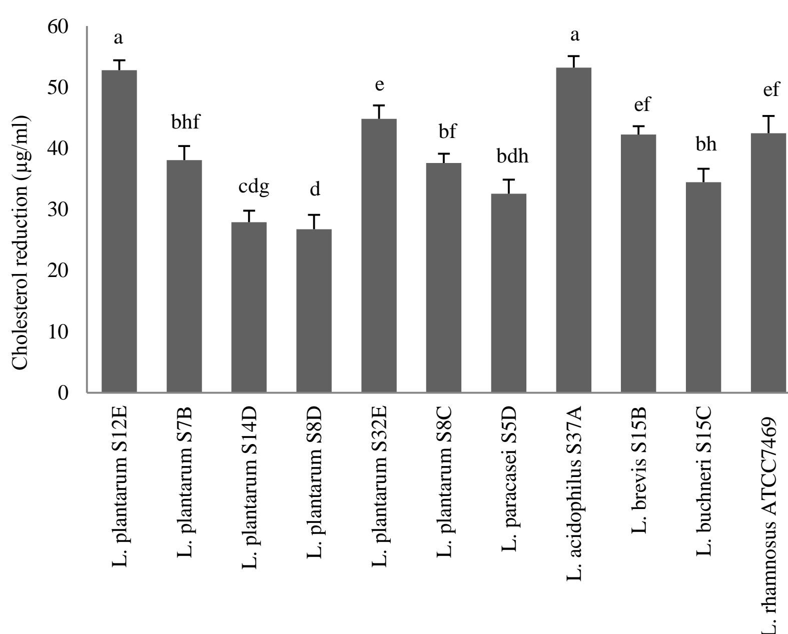

Fig. 2- The capacity of cholesterol reducing of selected *Lactobacillus* strains. Different letters (a-h) indicate significantly difference ($P < 0.05$)

Antimicrobial activity and antibiotic susceptibility: The antimicrobial properties of lactic acid bacteria can be attributed to the bioactive compounds such as organic acids, short chain fatty acids and bacteriocins (1). A broad spectrum of the inhibitory effect of supernatants derived from the 10 tested *Lactobacillus* strains was seen against the target pathogens (Table 2). The inhibition potency can be grouped to negative (–; no growth-free inhibition zone), mild (+; ZOI: 0–2.0 mm), strong (++; ZOI: 2.1–4.0 mm), and very strong (+++; ZOI: >4.0 mm). Accordingly, strains S32E, S37A and S15C, and strains S12E, S14D and S15B were categorized as strong and very strong inhibitors against *L. monocytogenes*, respectively. In addition, strains S12E, S7B, S14D, S32E and S15B, and strains S8C, S5D, S37A, S15C and S8D exhibited strong and mild antimicrobial activity against *S. aureus*, respectively. However, strain S8D had no antimicrobial inhibitory effect on *S. aureus*.

It was found that the antimicrobial activity of *Lactobacillus* strains against the tested Gram negative bacteria (*S. typhimurium* and *E. coli* O157:H7) was weaker than what was observed in case of the Gram positive bacteria. Three strains, i.e., S7B, S8D, and S5D showed no detectable inhibition against *S. typhimurium*, and four strains, i.e., S8D, S8C, S5D, and S15C against *E. coli* O157:H7. These findings are somewhat in agreement with data from previous reports by other authors (29, 30). In general, the antimicrobial activity of *Lactobacillus* strains is in a species- and strain-dependent manner.

The increasing occurrence of antibiotic–resistance as a complex phenomenon is due to interactions between human, animals, bacteria, drugs and environment (31). According to the guidelines of European Food Safety Authority (EFSA), fermented and probiotic foods which contain antibiotic resistant strains should not be consumed by a human (32). Therefore, assessment of antibiotics susceptibility of lactic acid bacteria as suitable probiotic candidates is inevitable. According to the known breakpoints of CLSI (17) for antibiotic susceptibility testing of microorganisms, results should be categorized as susceptible (ZOI: ≥ 20 mm), intermediate (ZOI: 15–19 mm ≥ 20 mm), and resistant (ZOI: ≥ 20 mm). As shown in Table 2, *L. acidophilus* S37A, *L. plantarum* S12E, and *L. plantarum* S32E were sensitive against 5, 4, and 4 antibiotics, respectively. In addition, *L. parcasei* S5D was the most resistant strain against the tested antibiotics (Table 2). All in all, most antibiotic resistibility was detected for ciprofloxacin and gentamicin. Intrinsic resistance to aminoglycosides including gentamicin can be attributed to the inability of lactobacilli to uptake the antibiotics due to the absence of cytochrome-mediated electron transport (33). Also, intrinsic resistance of lactobacilli to ciprofloxacin as an inhibitor of DNA replication can result from properties of the wall structure, permeability, or an impotent efflux mechanism (34).

Antioxidative activity of lactobacilli strains

In addition to the potential probiotic properties of lactic acid bacteria, considerable attention has recently been centered on their antioxidative capacity (35, 36). Regarding distinct mechanisms of antioxidant compounds to exert their antioxidative effect, it was reasonably suggested that more than one isolated method should be used to fully evaluate the antioxidative activity (10). Therefore, four separate assays were applied to evaluate this capacity.

Scavenging of DPPH radicals: The intracellular cell-free extracts or intact cells of the lactobacilli strains were found to scavenge DPPH radicals (Table 3). The highest scavenging activity of intracellular cell-free extracts (27.99 ± 1.77%) or intact cells (35.90 ± 2.13%) was observed in *L. parcasei* S5D. In general, the scavenging activity of intact cells was more than that of the intracellular cell-free extracts. In this regard, nine of the ten strains were statistically different ($P<0.05$). It was reported that cell surface components such as proteins and polysaccharides of lactobacilli have a positive effect on the DPPH scavenging activity (6). Therefore, the removal of these components that would occur following centrifugation the disrupted cells could result in a sharp decline of DDPH radical scavenging activity of intracellular cell-free extracts. Similarly, results of the investigations of other authors indicated that the DDPH radical scavenging capacity of intact cells of several lactic acid bacteria was higher as compared to the intracellular cell-free extracts (18, 37, 38).

Table 2- Antimicrobial activity against target pathogens and antibiotic susceptibility pattern of selected *Lactobacillus* strains.

Strain	Antimicrobial activity[a]				Antibiotic susceptibility[b]							
	Target pathogen				Interpretation							
	L. monocytogenes	*S. aureus*	*S. typhimurium*	*E. coli* O157:H7	Penicillin G	Ampicillin	Cefixime	Gentamicin	Tetracycline	Chloramphenicol	Ciprofloxacin	Clindamycin
L. plantarum S12E	+++	++	+	+	I	S	S	I	I	S	I	S
L. plantarum S7B	+	++	−	+	R	I	S	R	R	I	R	S
L. plantarum S14D	+++	++	++	+	S	R	I	I	I	S	R	I
L. plantarum S8D	+	−	−	−	I	S	S	I	R	R	S	I
L. plantarum S32E	++	++	+	+	S	R	S	I	S	S	I	I
L. pentosus S8C	+	+	+	−	I	I	S	R	R	S	I	I
L. parcasei S5D	+	+	−	−	R	R	I	R	I	I	R	S
L. acidophilus S37A	++	+	+	+	S	S	I	I	I	S	R	S
L. brevis S15B	+++	++	+	++	R	I	S	R	S	S	R	R
L. buchneri S15C	++	+	+	−	S	I	S	S	R	R	R	I
L. rhamnosus ATCC 7469	++	++	+	+	S	I	S	I	R	S	I	R

[a] –: no zone of inhibition; +: zone of inhibition 0–2.0 mm; ++: zone of inhibition 2.1 to 4.0 mm; +++: zone of inhibition >4.0 mm.
[b] S: sensitive; I: intermediate; R: resistant.

Scavenging of hydroxyl radicals: The hydroxyl radical is known as the most reactive oxygen species causing DNA damage and lipid peroxidation (39). As shown in Table 3, intracellular cell-free extracts or intact cells of the lactobacilli strains exhibited various levels of scavenging activities against hydroxyl radicals. The scavenging activity values ranged from 24.04 ± 0.85 to 33.50 ± 1.22% and 11.70 ± 0.49 to 23.31 ± 0.84 % for intracellular cell-free extracts or intact cells, respectively. Intracellular cell-free extracts of *L. plantarum* S8D extracts and intact cells of *L. parcasei* S5D had the highest hydroxyl radical scavenging activities, respectively.

In contrast to the results of DPPH scavenging activity assay, it was found that the intracellular cell-free extracts of all *Lactobacillus* strains can significantly increase ($P<0.05$) the scavenging activity of hydroxyl radicals in comparison with intact cells. This can be attributed to the presence of certain intracellular components such as GSH. In fact, intracellular GSH is known to have an important role to scavenge hydroxyl radicals (39, 40). According to Kullisaar et al. (40), the hydroxyl radical eliminating ability of lactobacilli directly depends on their GSH content. It was recognized that the strains with higher hydroxyl radical scavenging capacity including *L. parcasei* S5D, *L. acidophilus* S37A, and *L. plantarum* S8D showed higher GSH activity in later stages of this investigation (Table 3).

Table 3- Antioxidant capacities of the selected *Lactobacillus* strains.

Strain	Antioxidative activity							
	DPPH scavenging activity (%)		Hydroxyl radical scavenging activity (%)		SOD activity (U/mg)	Glutathione activity (μmol/ml)		
	Intracellular cell-free extract	Intact cell	Intracellular cell-free extract	Intact cell	Intracellular cell-free extract	Intracellular cell-free extract		
						TGSH	GSH	GSSG
L. plantarum S12E	$19.59 \pm 1.57^{abcfg\ A}$	$25.44 \pm 1.13^{ab\ B}$	$23.45 \pm 0.87^{ab\ A}$	$14.62 \pm 0.61^{ace\ B}$	ND	0.42 ± 0.07^{ad}	0.28 ± 0.08^{a}	0.13 ± 0.0^{a}
L. plantarum S7B	$20.60 \pm 1.34^{abcg\ A}$	$25.62 \pm 0.94^{a\ B}$	$26.95 \pm 1.64^{ae\ A}$	$12.79 \pm 0.40^{ac\ B}$	ND	0.71 ± 0.05^{ab}	0.57 ± 0.05^{be}	0.14 ± 0.01^{a}
L. plantarum S14D	$18.29 \pm 1.22^{acf\ A}$	$20.85 \pm 1.54^{bd\ A}$	$22.15 \pm 1.02^{bdg\ A}$	$11.70 \pm 0.49^{a\ B}$	ND	0.86 ± 0.11^{bf}	0.68 ± 0.07^{b}	0.18 ± 0.04^{ac}
L. plantarum S8D	$23.96 \pm 2.62^{beg\ A}$	$28.96 \pm 1.28^{ac\ B}$	$33.50 \pm 1.22^{c\ A}$	$22.06 \pm 1.06^{b\ B}$	ND	1.28 ± 0.15^{c}	0.92 ± 0.08^{cf}	0.37 ± 0.08^{b}
L. plantarum S32E	$16.32 \pm 2.04^{cd\ A}$	$33.49 \pm 1.02^{ce\ B}$	$20.04 \pm 1.32^{b\ A}$	$11.78 \pm 1.02^{a\ B}$	0.14 ± 0.01^{a}	0.32 ± 0.03^{d}	0.22 ± 0.03^{a}	0.11 ± 0.01^{a}
L. plantarum S8C	$12.31 \pm 1.24^{d\ A}$	$19.21 \pm 1.05^{d\ B}$	$24.04 \pm 0.85^{ad\ A}$	$14.96 \pm 1.44^{ce\ B}$	ND	0.26 ± 0.03^{d}	0.24 ± 0.03^{a}	-
L. parcasei S5D	$27.99 \pm 1.77^{e\ A}$	$35.90 \pm 2.13^{e\ B}$	$29.23 \pm 1.03^{ef\ A}$	$23.31 \pm 0.84^{b\ B}$	0.20 ± 0.02^{b}	1.83 ± 0.11^{e}	1.49 ± 0.06^{d}	0.34 ± 0.07^{bc}
L. acidophilus S37A	$23.41 \pm 1.63^{abe\ A}$	$34.47 \pm 2.74^{e\ B}$	$31.63 \pm 1.31^{cf\ A}$	$18.21 \pm 1.63^{d\ B}$	0.11 ± 0.01^{c}	1.85 ± 0.12^{e}	1.42 ± 0.03^{d}	0.43 ± 0.14^{b}
L. brevis S15B	$14.94 \pm 2.10^{df\ A}$	$23.42 \pm 2.14^{ad\ B}$	$29.02 \pm 0.87^{ef\ A}$	$18.53 \pm 0.81^{d\ B}$	ND	1.36 ± 0.14^{c}	1.02 ± 0.09^{c}	0.34 ± 0.07^{bc}
L. buchneri S15C	$18.80 \pm 1.45^{abcf\ A}$	$26.91 \pm 1.26^{a\ B}$	$24.78 \pm 0.91^{agh\ A}$	$17.10 \pm 1.12^{de\ B}$	ND	0.49 ± 0.04^{ad}	0.39 ± 0.02^{ae}	0.1 ± 0.02^{a}
L. rhamnosus ATCC 7469	$24.11 \pm 1.97^{eg\ A}$	$32.91 \pm 1.56^{ce\ B}$	$28.20 \pm 1.76^{efh\ A}$	$18.84 \pm 1.52^{d\ B}$	0.15 ± 0.01^{a}	1.10 ± 0.17^{cf}	0.75 ± 0.13^{bf}	0.35 ± 0.04^{bc}

Mean values within a column with unlike lower case superscript letters (a-h) are significantly different ($P<0.05$). Mean values within a row with unlike upper case superscript letters (A, B) are significantly different between intracellular cell-free extract and intact cell ($P<0.05$).

Superoxide dismutase (SOD) activity: In general, most lactobacilli lack intracellular SOD enzymes which, in turn, can significantly increase their resistivity to oxidative stress (41). However, a number of workers have recognized varying levels of SOD activities in some lactobacilli strains (6, 35, 42). The results indicated that only 3 of 10 strains were able to cause SOD activity (Table 3). Accordingly, SOD activity of 0.20 ± 0.02, 0.14 ± 0.01, and 0.11 ± 0.01 U/mg was measured for *L. parcasei* S5D, *L. plantarum* S32E, and *L. acidophilus* S37A.

Glutathione content: Total glutathione (TGSH) and glutathione (GSH), and oxidized glutathione (GSSG) concentrations are presented in Table 3. A range of concentration from 0.26 ± 0.03 to 1.85 ± 0.12 to μmol/ml was measured for TGSH. In addition, GSH and GSSG contents ranged from 0.24 ± 0.03 to 1.49 ± 0.06 μmol/ml and from 0.1 ± 0.02 to 0 μmol/ml 0.43 ± 0.14 μmol/ml, respectively. Probiotics can produce several metabolites with antioxidant capacity such as GSH. This major cellular metabolite acts as a non-enzymatic antioxidant to eliminate hydrogen peroxide, hydroxyl radicals, and peroxynitrite (43, 44). The positive correlation between antioxidative activity and cellular GSH has been reported (45).

In conclusion, out of the 101 isolates, 20 were exhibited to have the highest acid and bile salts tolerability and were, thus, assayed by molecular tools. As a result, ten isolates were confirmed to belong to the *Lactobacillus* genus. Further 16S rRNA gene sequencing of isolates was used to characterize them at the species level. Potential probiotic characteristics of some of the tested strains, in particular *L. acidophilus* S37A and *L. plantarum* S32E,

were close or even better than the reference probiotic strain *L. rhamnosus* ATCC 7469. Regarding the antioxidative activities of the strains, *L. plantarum* S8D, *L. parcasei* S5D, *L. acidophilus* S37A, and *L. brevis* S15B were superior to the other strains. However, further *in vivo* investigations are required to explore the probiotic properties and the health benefit or antioxidative potencies of these strains. All in all, the artisanal Poosti cheese could be considered as a potential source of probiotics.

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