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Full Length Research Paper

Traditional Iranian dairy products: A source of potential probiotic lactobacilli

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A varied climate in Iran makes the production of a wide range of dairy products possible. The aim of this study was to isolate and identify new potential probiotic lactobacilli from traditional Iran dairy products. The isolates were screened for their probiotic potential activities, including acid and bile resistance, antagonistic activity and cholesterol removal. Screening of acid and bile tolerant strains from 14 different samples led to the identification of 20 isolates of *Lactobacillus* spp. Most promising strains which assimilated more than 75% of the cholesterol in the medium and/or exhibited high inhibition value against pathogen identified based on 16S rDNA sequence. *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus casei* isolated from yogurt and cheese exhibited both high cholesterol assimilation and antagonist activity. These results suggested that, Iranian indigenous lactobacilli have potential as probiotics, and they might also be good candidates to be used as probiotic carrier or functional foods.

Key words: Traditional dairy, probiotic, cholesterol assimilation, antagonist activity.

INTRODUCTION

The relationship between dairy foods and health effects has been investigated for many years (Heller, 2001). During recent years, numerous studies have been undertaken to obtain scientific evidence for beneficial effects of fermented dairy products containing specific probiotic strains (Ouwehand et al., 2002; Tuomola et al., 2002). Probiotics have been defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Araya et al., 2002; WHO/FAO, 2002). Traditional fermented dairy products are, together with contents in the gastro-intestinal tract, the main sources for isolation of potential probiotic organisms (Ambadoyiannis et al., 2005). The varied climate in Iran makes the production of a wide range of dairy products possible, particularly in rural regions. In this perspective, traditional Lighvan dairy products such as different kinds of cheese, yogurt, long life yogurt, kashk, could play a role in the selection of new potential probiotic bacteria. Not only because of their physicochemical properties caused by the use of raw cow's milk as source of fermenting microorganisms, but also due to the presence of high levels of viable lactic acid bacteria at the moment of consumption. Lighvan dairy products being produced by small scale artisan manufacturers, using milk from sheep and cows bred in semi-wild conditions and enjoy great popularity in Iran. Therefore, we focused on isolating lactobacilli from traditional Lighvan dairy products and to assess their potential as probiotics.

The main *in vitro* selection criteria for potential probiotic strains are acid and bile resistance activities, indicating the ability of the organism to survive the passage through the gastrointestinal tract (Jacobsen et al., 1999; Pennacchia et al., 2004; Garabal et al., 2008). Production of antimicrobial compounds, such as bacteriocins, lactic and acetic acid and competition for nutrients and adhesion sites may contribute to the control of intestinal pathogens (Tagg et al., 1976; Lee et al., 2000; Candela et al., 2008). Furthermore, milk fermented by certain

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Lactobacillus strains has been shown to decrease, total serum cholesterol and low-density lipoprotein (LDL) cholesterol (Pereira and Gibson 2002; Liong and Shah 2005; Ataie-Jafari et al., 2009). Finally, proper identification is of prime importance as indicator for safety and traceability (Morelli, 2000; Pereira et al., 2002).

The aim of the current study was therefore to isolate and identify new potential probiotic Lactobacillus isolates with acid and bile tolerance by screening a range of traditional Lighvan dairy products and to assess their potential beneficial effects including antagonistic and cholesterol reduction activity.

MATERIALS AND METHODS

Selective screening and isolation

Fourteen samples (20 g each) of traditional fermented dairy products, including different kinds of yogurt, cheese, fermented milk, dough and kashk, (some of these products were prepared from raw milk) were inoculated into 200 ml of MRS broth (Fluka and catalogue no. 69966). To decrease the risk of missing strains, all samples were centrifuged after each step. After anaerobic incubation at 37°C for 24 h, 30 ml of the broth was centrifuged at 1000 xg for 15 min and the pellet was resuspended into 10 ml of PBS adjusted to pH 2.5 with HCl. After 2 h of anaerobic incubation at 37°C, the medium was centrifuged at 4000 xg for 20 min, the pellet resuspended in ringer solution and plated on MRS agar plate (Pennacchia et al., 2004).

In order to avoid preliminary time consuming isolation steps and interference of yeast, lactobacilli colonies were selected on MRS agar containing nystatin (Gardiner et al., 2004). The plates were incubated under anaerobic conditions at 37°C for 48 to 72 h. Colonies, characterized with different morphologies, were isolated and purified on MRS agar. All isolates were examined by microscopy after gram staining and catalase reaction. Gram positive, catalase negative rods were cultured in MRS broth and stored at -80°C (Succi et al., 2005). The main purpose of this study was screened for the most promising isolates in each step were selected for further evaluation in the next step.

Acid resistance

Acid resistance was determined by comparing survival in PBS at pH 6.0 and 2.5 as described by Pennacchia (Pennacchia et al., 2004).

Bile tolerance

The most acid resistant isolates were selected for bile tolerance evaluation. Bile resistance was determined as described by Gilliland (Gilliland et al., 1984). In short, growth was determined spectrophotometrically (600 nm) in MRS containing 0.3% bile salts (Sigma and catalogue no. B8381) and compared to bile salt-free MRS. Oxgall was used in this study, as it contained a combination of both conjugated and deconjugated bile acids. Growth delay was used as a measure of tolerance (Gilliland et al., 1984; Saavedra et al., 2003).

Identification of the bacterial isolates

The isolates were identified according to their morphological, cultural, physiological and biochemical characteristics based on Bergey's Manual (Garrity, 2005). The used tests were: Gram reaction; production of catalase, cytochrome oxidase, hydrogen peroxide, growth at 15 and 45°C in 1 week; acid production from carbohydrates (1% w/v) and ammonia production from arginine (Muyanja et al., 2003).

16S rDNA amplification and sequencing

Genomic DNA from MRS broth cultures were extracted by phenol extraction method as reported by Araújo and Sambrook (Araujo et al., 2004; Sambrook et al., 1989). PCR-mediated amplification of the complete 16S rDNA was carried out in a Gradient Master Thermocycler (Bio Rad). All reagents if not indicated otherwise were purchased from Fermentase Life Science, Germany. The amplification conditions were as follows: 1 µl genomic DNA, 10 µl 10 × reaction buffer, 200 nM each of the four deoxynucleotides, 3 U Taq polymerase, 20 pmol of each primer (Interactiva) (616V, 5`-AGAGTTTGATYMTGGCTCAG-3`; 630R, 5`-CAKAAAGGAGGTGATCC-3`), dH_2O to a final volume of 100 µl. The PCR conditions were: (94°C/2 min)1×, (94°C/45 s, 52°C/1 min, 72°C/30 s) 30×, (94°C/1 min, 72°C/4 min) 1× (Ehrmann et al., 2003). Amplified fragments were cloned using TA Cloning system (T-easy cloning vector, Promega) and transformed to DH5α by heat shock method (Sambrook et al., 1989). Plasmids were extracted using Core-one plasmid isolation kit Macrogen (Seoul, South Korea). Restriction fragments analysis performed for cloning verification using EcoR I restriction enzymes according to Promega company procedure. Selected plasmids sequenced by Macrogene (Seoul, South Korea). Sequences were BLAST in GenBank database (www.ncbi.nlm.nih.gov) for species assignment.

Phylogenetic analysis

The complete 16S rDNA sequence of selected strains were fitted into alignments of almost complete primary structures available in NCBI database Nucleotide collection (nr/nt) using Megablast, optimize for highly similar sequences. Maximum parsimony and maximum-likelihood methods were applied for tree reconstructions. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007).

Screening of lactobacilli for antimicrobial activities

All isolates selected based on their acid and bile resistances were assessed for their antagonistic activity against various strains of indicator pathogens using bi-layers and well-diffusion methods. Indicators used included *Escherichia coli* (ATCC 2143), *Staphylococcus aureus* (ATCC 64542), *Listeria monocytogenes* (ATCC 345), *Yersinia entrocolitica* (ATCC 25673) and *Listeria innocua* (DSMZ 20649). Briefly, 2 µl of active (overnight) MRS culture of selected Lactobacillus isolates were spotted at the center of MRS soft agar plates (containing 0.7% agar). Plates were dried for 30 min at room temperature and incubated for 48 h. After growth, the colonies were killed by exposure to chloroform vapor for 1 h and the colonies were removed from the surface of the plates with cotton. The plates were overlaid with BHI (Fluka and catalogue no. 53286) soft agar (containing 0.7% agar) inoculated with 0.5% of 24 h broth culture of an indicator strain. The plates were left at 5°C for 2 h to allow diffusion of the tested metabolite, and then incubated aerobically for 24 h at 37°C. Plates were examined for an inhibition zone in the bacterial lawn just above the spots and expressed as diameter (Upreti and Hinsdill, 1973; El-Naggar, 2004). Total inhibition value for each Lactobacillus isolates was determine by making percent value of entirety; all inhibition zones

against the five indicator bacteria minus from 30 (plate size 6 cm × 5 indicator bacteria). Experiments were performed in triplicate.

Lactobacillus isolates showing antagonistic activity were further studied for the production of inhibitory substances into their culture medium, using the agar diffusion method described by Tagg (Tagg et al., 1976). An overnight MRS culture of lactobacilli was centrifuged at 7000 xg for 10 min and the pH of half of supernatant were adjusted to 7.0 ± 0.1 with 1 mol L^{-1}NaOH and filtered through a 0.22 µl pore-size filter. The other half of the supernatant was filtered without any change in pH. The supernatants were tested directly by agar diffusion method. Briefly, 25 ml of MRS soft agar, containing 0.7% (w/v) agar seeded with the indicator bacteria at a final concentration of about 106 CFU/ml was allowed to solidify at room temperature. Wells (6 mm) were cut in the solidified agar and filled with 100 µl of the test supernatants. The plates were left at 5°C for 2 h to allow diffusion of the supernatant and subsequently incubated aerobically for 24 h at 37°C. Absence or presence of any inhibitory zone was recorded. (Schillinger and Lucke, 1989; Strompfová et al., 2004).

Cholesterol removal

Water-soluble cholesterol (polyoxyethanyl cholesteryl sebacate) (Sigma and catalogue no. 698717) was filter-sterilized and added to the broth at a final concentration of 300 µg/ ml, inoculated with each selected isolate (at 1%), and incubated anaerobically at 37 °C for 24 h (Liong and Shah, 2005). After the incubation period, cells were centrifuged and the remaining cholesterol concentration in the broth was determined using a modified colorimetric method as described by Rudel (Rudel and Morris, 1973). 1 ml of the aliquot was mixed with 1 ml KOH (33% w/v) and 2 ml absolute ethanol, vortexed for 1 min, and incubated 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. 1 ml of the hexane layer was transferred into a glass tube and evaporated under nitrogen. The residue was immediately dissolved in 2 ml of o-phthalaldehyde reagent. After complete mixing, 0.5 ml of concentrated sulfuric acid was added and the mixture was vortexed for 1 min. Absorbance was read at 550 nm after 10 min. All experiments were performed in triplicate.

Statistical analysis

Scores were summed for each isolate and the data produced were analyzed statistically using the ANOVA technique. Statistical significance was attributed to $P \leq 0.05$.

RESULTS

Screening of acid tolerant strains in presence of acidic phosphate buffer (pH=2.5) led to the cultivation of 66 isolates of *Lactobacillus* spp. from 14 different fermented dairy products. Most of them were isolated from cheese and yogurt; 34 and 14 respectively. All isolates survived exposure to PBS at pH 6, with negligible loss of viability, results not shown. However, only 18 isolates exhibited resistance to pH 2.5, indicated by survival at levels of at least 108 CFU/ml. Thirty strains were judged resistant to pH 2.5 as indicated by survival at levels of 106-1010 CFU/ml (Table 1). The remaining isolates (36) were considered sensitive and were excluded from further studies, data not shown. The 30 acid tolerant isolates were tested for their bile tolerance as determined by the delay of growth (D) in MRS broth supplemented with bile as compared to unsupplemented MRS. The delay of growth ranged from 5 min to more than 3 h. The 20 acid and bile tolerate isolates, were identified based on Bergey`s manual (Table 1).

The levels of cholesterol assimilation during 24 h of growth varied considerably among the tested isolates and ranged from 34 to 276 µg/ml. Eleven isolates assimilated more than 50% of the cholesterol in the medium and seven isolates, *L. casei* y2c4, *Lactobacillus farieminis* C4i2, *Lactobacillus acidophilus* Y2b9, *L. casei* Y2f3, *Lactobacillus alimentarium* Y1l4, *L. plantarum* C6m3 and *L. plantarum* C5i4 (preliminary identification based on morphology and biochemical properties), assimilated more than 75% cholesterol compared with the control (Table 1). The antagonistic activity exhibited by the most promising Lactobacillus isolates, as based on their acid and bile resistance, was evaluated by bi-layers and agar-well diffusion methods. Inhibition zones varied among strains and ranged from 0 to 6 cm in bi-layers assay. Based on these, data inhibition value defined and ranged from 15.7 to 88.7% (Table 2). When the neutralized supernatants of antagonistic strains were assessed directly by the agar-well diffusion method, no detectable signs of inhibition could be observed for any strains except C4i2 that showed 2±0.5 mm inhibition zone against *L. monocytogenes*. However, supernatants maintained at their original pH from five strains produced inhibition zones against some indicator bacteria (Table 3).

Seven strains assimilated more than 75% of the cholesterol in the medium and six strains exhibited strong antagonist activity against both Gram positive and negative bacteria, identified based on 16S rDNA sequence. However, three strains of them *L. casei* (Y2c4), *L. farieminis* (C4i2) and *L. plantarum* (C5i4) exhibited both high cholesterol assimilation and antagonist activity. These 10 most promising isolates were identified by 16S rDNA sequences and were deposited in the NCBI nucleotide sequence databases under the accession numbers as shown in Table 4. The phylogenetic position is shown in Figure 1.

DISCUSSION

Due to the varied climate in Iran, there is a wide variety of traditional dairy products in the countries rural areas. These products are made from unpasteurized ewe's or cow's milk or mixtures of both. The Iranian consumer prefers to use traditional dairy products due to their excellent natural tastes and flavors. In this perspective, such products are potentially good candidates for isolating new strains of probiotics. Also, they can be considered as natural functional foods after comprehensive evaluation. Fermented dairy products are complex ecosystems dominated by lactic acid bacteria. Direct isolation of *Lactobacillus* spp. with interesting

Table 1. Biochemical identification, acid resistance, bile tolerance and cholesterol assimilation of *Lactobacillus* spp. isolates.

Isolate	*Lactobacillus* species C	Origin	Acid resistance (CFU/ml)b	Growth delay, 0.3% bile (min)	Cholesterol assimilation (µg/L)
Y1m4	*casei*	Yogurt	1.20×108 ±0.42	5±1.24	153±4.21
Y2f3	*casei*	Yogurt	5×108 ±0.42	15±1.63	263±3.03
C6m3	*plantarum*	Cheese	1.40×109±0.12	25±4.08	276±2.08
Y2b9	*acidophilus*	Yogurt	3×108 ±0.29	30±2.36	240±2.94
Y2l6	*casei*	Yogurt	3.60×106±0.14	30±2.36	141±3.68
C6m1	*jensenii*	Cheese	2.40×107 ±0.16	30±2.40	41±1.70
Y2b10	*salivarius*	Yogurt	2.20×109±0.43	40±4.08	161±2.86
Y2n2	*casei*	Yogurt	1.80×108±0.65	45±4.08	211±3.55
C2h1	*lactis*	Cheese	3.40×107±0.09	45±2.08	54±3.09
Y2c4	*casei*	Yogurt	4.50×108±0.45	50±6.23	225±2.15
Y1l4	*alimentarium*	Yogurt	6.70×108±0.45	60±4.08	266±4.09
D3b1	*plantarum*	Fermented milk	4.10×109±0.12	60±2.35	161±0.81
K1l4	*plantarum*	Kashk	3.60×109±0.21	60±2.35	113±1.69
C1d2	*lactis*	Cheese	1×1010±0.24	60±4.08	41±2.16
C6l2	*rhamnosus*	Cheese	4.70×106±0.18	60±1.69	34±2.61
D2d1	*plantarum*	Fermented milk	5.90×108±0.39	70±3.29	70±2.30
C4i2	*farieminis*	Cheese	1.80×109±0.18	90±5.27	240±4.08
K2l3	*agilis*	Kashk	1.70×107±0.53	90±2.94	99±0.81
Y2p3	*casei*	Yogurt	4.90×107±0.38	85±0.82	99±4.080
C5i4	*plantarum*	Cheese	2.50×109±0.34	90±4.08	226±1.70
C5b5	*NDa*	Cheese	1.30×109±0.33	150±3.56	ND
C5b4	*ND*	Cheese	3.80×108±0.16	150±4.08	ND
C6c4	*ND*	Cheese	5.50×106±0.41	>150	ND
C2h1	*ND*	Cheese	1.90×106±0.12	>150	ND
D2l1	*ND*	Fermented milk	3.60×106±0.15	>150	ND
D2n2	*ND*	Fermented milk	6.40×108±0.22	>150	ND
C5a9	*ND*	Cheese	5.30×107±0.17	>150	ND
B1c2	*ND*	Butter	1.90×106±0.22	>150	ND
C6k1	*ND*	Cheese	3.50×109±0.25	>150	ND
D3b1	*casei*	Fermented milk	4.60×109±0.53	>150	ND

ND; not determined ; Results are expressed as mean ± SEM; each data point is the average of 3 repeated measurements from 3 independently replicated experiments, “n = 3”. C Species identification based on morphology and biochemical properties.

functional traits from these products is complicated (Watanabe et al., 2008).

Since viability and survival of probiotic bacteria during passage through the stomach is an important parameter to reach the intestine and provide potentially beneficial effect, the first step of this study was focused on the selection of acid tolerant isolates. A simple rapid screening method was used to isolate acid tolerant bacteria from 14 different fermented dairy products. The pH value (2.5) used in this study for the selection of potentially probiotic strains is very stringent, even though it is not the most common pH value of the human stomach. In general, the most acid resistant isolates were originated from Sabalan cheese, fresh and long preserved Lighvan cheese, and yogurt compared with those isolates from fermented milk and kashk.

The next challenge for selection of probiotic survivors in gastrointestinal tract is exposure to bile salts in the upper part of the small intestine (Pennacchia et al., 2004; Pan et al., 2009). Oxgall was used, to study bile tolerance of the organisms by measuring of the lag phase in growth caused by the presence of bile in liquid medium (Morelli, 2000). Results showed that, the delay detected in the growth curve of Lactobacillus isolates challenged with oxgall, was strain-dependent, and not species-dependent. This has been earlier observed for other species of Lactobacillus isolates from humans, and strains intended for human consumption (Morelli, 2000). Of the 30 selected Lactobacillus isolates cultured in the presence of 0.3% bile salts, 20 isolates were able to grow with different levels of tolerance. There was substantial variability in resistance to bile salts among the

Table 2. Inhibition zone (mm)a among the isolate evaluated by bi-layers assay.

Strain code	*Y. enterocolitica*	*L. inocua*	*L. monocytogenes*	*S. aureus*	*E. coli*	Inhibition value (%)
Y1m4	0	58±2.3	21±1.6	27±2.6	22±2.8	43.0
Y2f3	10±2.5	40±2.4	28±1.7	0	30±2.5	28.1
C6m3	0	0	20±8.1	20 ±1.2	6±1.7	36.3
Y2b9	0	20±1.2	0	30±7.3	30±2.4	15.4
Y2l6	20±1.6	29±2.9	59±5.9	58±2.6	41±2.1	69.2
C6m1	0	0	21±2.4	5±1.2	30±2.9	18.7
Y2b10	20±1.3	0	0	58±3.5	20±0.4	26.1
Y2n2	0	0	15±2.9	59±0.9	10±4.5	28.1
C2h1	0	20±1.2	39±1.3	20±1.2	20±2.0	32.2
Y2c4	58±1.6	58±1.2	59±2.6	19±0.9	49±0.9	80.8
Y1l4	18±1.7	41±2.6	43±5.7	30±4.4	0	44.3
D3b1	25±4.0	55±7.0	56±4.1	18±3.2	57±2.4	70.2
K1l4	58±2.8	22±2.8	58±2.6	59±1.4	29±2.8	75.4
C1d2	57±4.7	35±1.2	39±0.8	39±1.4	49±0.8	73.0
C6l2	59±0.8	0	57±2.4	39±3.2	42±8.7	65.8
D2d1	22±2.4	10±0.8	40±1.3	0	15±2.1	29.0
C4i2	58±2.3	55±4.5	50±4.0	54±3.2	49±1.2	88.6
K2l3	0	59±1.8	38±6.2	20±2.1	34±3.0	50.5
Y2p3	58±4.5	59±0.9	51±1.6	10±2.4	0	60.1
C5i4	58±1.6	28±2.1	58±4.7	59±0.9	12±2.1	71.3

Results are expressed as mean ± SEM; each data point is the average of 3 repeated measurements from 3 independently replicated experiments; “n=3”.

Table 3. Inhibition zone (mm) among the isolates evaluated by the agar-well diffusion method (non-neutralized supernatants).

Strains code	*Y. enterocolitica*	*L. inoccua*	*L. monocytogen*	*S. aureus*,	*E. coli*,
C4i2	0	2±0.5	3±0.8	3±0.7	5±0.3
K2l3	0	0	1±0.1	0	1±0.2
Y1l4	0	0	3±0.4	3±0.9	0
C5i4	2±0.7	1±0.2	3±0.8	2±1	2±0.3
C6l2	0	0	2±0.5	3±0.6	0
C5i4	0	0	3±0.9	0	0

Results are expressed as mean±SEM; each data point is the average of 3 repeated measurements from 3 independent experiments; n=3.

Table 4. Species identification by sequencing of the 16s rDNA.

Isolate	Species	Similarities (%)	Accession number
Y2f3	*Lactobacillus brevis*	99	GQ423768
C6m3	*Lactobacillus* plantarum	99	GQ423760
Y2b9	*Lactobacillus pentosus*	98	GQ423764
Y2c4	*Lactobacillus plantarum*	97	GQ423755
Y1l4	*Lactobacillus brevis*	98	GQ423766
D3b1	*Lactobacillus brevis*	100	GQ423757
K1l4	*Lactobacillus plantarum*	96	GQ423756
C1d2	*Lactobacillus plantarum*	98	GQ423761
C4i2	*Lactobacillus brevis*	100	GQ423758
C5i4	*Lactobacillus casei*	98	GQ423759

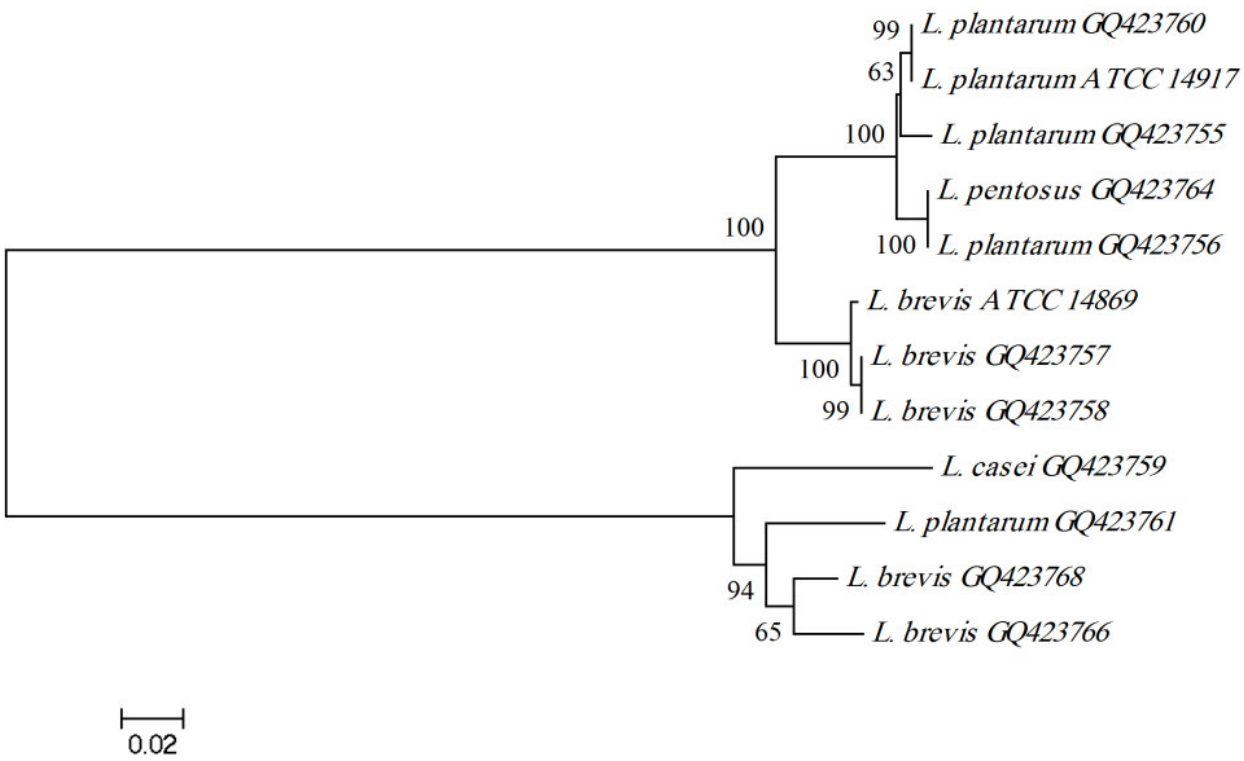

Figure 1. Phylogenetic tree based on the 16S rDNA sequencing of isolates. A phylogenetic tree based on the 16S rDNA sequencing of isolates and other reference strains. The tree was constructed by using the neighbor-joining method .Values at the base of clusters are bootstrap values. The bar indicates a genetic distance of 0.02.

Lactobacillus isolates and all the strains tested showed delayed growth, compared to unsupplemented MRS. Similar results were reported in other studies on several species of Lactobacillus (Gilliland et al., 1984; Chateau et al., 1994; Erkkilä and Petäjä, 2000; Succi et al., 2005). At the tested bile acid concentration, 8 isolates did not grow at all.

Elevated levels of certain blood lipids have been reported as the principal cause of cardiovascular disease, disabilities and even death (Pekkanen et al., 1990). Various approaches have been used to alleviate this issue, including the use of probiotics and/or prebiotics. In this study, we have examined the cholesterol assimilation abilities of the twenty Lactobacillus isolates that were acid and bile resistant. Some of these strains assimilated more than 200 µg/L cholesterol from media that is much more higher in comparison to what Liong and Pereira reported, 12.03 to 32.25 µg/ml and 0.4 to 47 µg/ml (Pereira et al., 2002; Liong et al., 2005). Most of the high cholesterol strains (more than 200 µg/ml) were isolated from long persevered yogurts and different Lighvan cheeses. No correlation was observed between oxgall tolerance and cholesterol assimilation. In other words, strains with the high bile tolerance did not necessarily assimilate more cholesterol than those with the lower tolerance. This contrasts findings by Liong whom reported that, strains showing greater tolerance toward deconjugated bile exhibited higher cholesterol assimilation (Liong et al., 2005).

The potential probiotic isolates were screened for their antagonistic activity against various strains of indicators using bilayers and well-diffusion methods. Based on bilayers data, an inhibition value for each strain was defined. In this method beside potential bacteriocin production, sugar fermentation followed by a reduction in pH due to the production of lactic and other organic acids is an important factor for the inhibition growth of undesired microorganisms. Six isolates, *L. casei*(D3b1), *L. plantarum* (C5i4), *L. casei* (Y2c4), *L. plantarum* (K1l4), *L.lactis* (C1d2), *L.farieminis* (C4i2) exhibited strong and four isolates, *Lactobacillus rhamnosus* (C6l2), *L. casei* (Y2p3), *L. alimentarium* (Y1l4), *L. casei* (Y2l6), had moderate antagonistic activity against both Gram positive and negative bacteria. The antagonistic activity exhibited by different Lactobacillus strains was further evaluated by the well-diffusion method. Most isolates did not inhibit indicator growth when the neutralized supernatants were applied directly to the agar diffusion test. However, non-neutralized supernatants of five isolates inhibited growth of some indicator bacteria. Based on these results, it is most likely that antagonistic activity is caused by production of organic acids and reduction of pH;

although, inactivation of a bacteriocin during neutralization cannot be ruled out. It is also possible that antimicrobial substances are membrane associated.

All isolates were preliminarily identified by phenotypic methods and the most promising isolates were identified by 16S rDNA gene sequencing. Our results demonstrate disagreement between the two procedures except for two *L. plantarum* isolates C6m3 and K1l4 isolated from cheese and kashk. Similar observations have been reported highlighting the importance of identification by molecular methods (Tannock et al., 1999).

Conclusion

We isolated 20 Lactobacillus strains capable of surviving the pH of the stomach and the environment of the intestine, which makes them potential probiotics. Seven isolates were able to assimilate 75% cholesterol from media compared with the control and six isolates had powerful antagonist activity. Lactobacillus originally isolated from traditional fermented dairy products are probably the most suitable candidates for inclusion, as probiotics into these foods, because they are well adapted to the conditions and may therefore be more competitive than probiotics from other sources.

These isolates do require further *in vitro* and *in vivo* investigations and specific studies on their technological properties in dairy fermentation. Moreover, before the suitable strains can be eventually used as probiotic starter cultures in novel dairy fermented products, their potential health benefits need to be investigated.

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REFERENCES

Ambadoyiannis G, Hatzikamari M, Litopoulou-Tzanetaki E, Tzanetakis N (2005). Probiotic and technological properties of enterococci isolates from infants and cheese. Food Biotechnol., 18: 307-325.

Araújo WL, Angellis DA, Azevedo JL (2004). Direct RAPD evaluation of bacteria without conventional DNA extraction. Brazilian Arch. Biol. Technol., 47: 375-380.

Araya M, Morelli L, Reid G, Sanders ME, Stanton C, Pineiro M, Embarek PB (2002). Guidelines for the evaluation of probiotics in food. Joint FAO/WHO Working Group Report Drafting Guidelines Eval. Probiotics Food, London (Ontario, Canada), 30: 1–11.

Ataie-Jafari A, Larijani B, Alavi Majd H, Tahbaz F (2009). Cholesterol-lowering effect of probiotic yogurt in comparison with ordinary yogurt in mildly to moderately hypercholesterolemic subjects. Ann. Nutr. Metabol., 54: 22-27.

Candela M, Perna F, Carnevali P, Vitali B, Ciati R, Gionchetti P, Rizzello F, Campieri M, Brigidi P (2008). Interaction of probiotic Lactobacillus and Bifidobacterium strains with human intestinal epithelial cells: adhesion properties, competition against enteropathogens and modulation of IL-8 production. Int. J. Food Microbiol., 125: 286-292.

Chateau N, Deschamps AM, Sassi AH (1994). Heterogeneity of bile salts resistance in the Lactobacin isolates of consortium. Lett. Appl. Microbiol., 18: 42-44.

Ehrmann MA, Muller MRA, Vogel RF (2003). Molecular analysis of sourdough reveals *Lactobacillus mindensis* spp. nov. Int. J. Syst. Evol. Microbiol., 53: 7-13.

El-Naggar MYM (2004). Comparative study of probiotic cultures to control the growth of *Escherichia coli* O157: H7 and *Salmonella typhimurium*. Biotechnol. (Pakistan). 2: 173-180.

Erkkilä S, Petäjä E (2000). Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. Meat Sci., 55: 297-300.

Garabal JI, Rodríguez-Alonso P, Centeno JA (2008). Characterization of lactic acid bacteria isolated from raw cows' milk cheeses currently produced in Galicia (NW Spain). LWT-Food Sci. Technol., 41: 1452-1458.

Gardiner GE, Casey PG, Casey G, Lynch PB, Lawlor PG, Hill C, Fitzgerald GF, Stanton C, Ross RP (2004). Relative ability of orally administered *Lactobacillus murinus* to predominate and persist in the porcine gastrointestinal tract. Appl. Environ. Microbiol., 70: 18-29.

Garrity GM (2005). Bergey's manual of systematic bacteriology Springer.

Gilliland SE, Staley TE, Bush LJ (1984). Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. J. Dairy Sci., 67: 3045-3050.

Gilliland SE, Walker DK (1990). Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. J. Dairy Sci., 73: 905-911.

Heller KJ (2001). Probiotic bacteria in fermented foods: Product characteristics and starter organisms. Am. J. Clin. Nutri., 73: 374-379.

Jacobsen CN, Rosenfeldt-Nielsen V, Hayford AE, Moller PL, Michaelsen KF, Paerregaard A, Sandstrom B, Tvede M, Jakobsen M (1999). Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. Appl. Environ. Microbiol., 65: 4949-4956.

Lee YK, Lim CY, Teng WL, Ouwehand AC, Tuomola EM, Salminen S (2000). Quantitative approach in the study of adhesion of lactic acid bacteria to intestinal cells and their competition with enterobacteria. Appl. Environ. Microbiol., 66: 3692-3697.

Liong MT, Shah NP (2005). Acid and bile tolerance and cholesterol removal ability of lactobacilli strains. J. Dairy Sci., 88: 55-66.

Liong MT, Shah NP (2005). Optimization of cholesterol removal by probiotics in the presence of prebiotics by using a response surface method. Appl. Environ. Microbiol., 71: 1745-1753.

Morelli L (2000). *In vitro* selection of probiotic lactobacilli: A critical appraisal. Curr. Issues Intestinal Microbiol., 1: 59-67.

Muyanja C, Narvhus JA, Treimo J, Langsrud T (2003). Isolation, characterisation and identification of lactic acid bacteria from bushera: A Ugandan traditional fermented beverage. Int. J. Food Microbiol., 80: 201-210.

Ouwehand AC, Salminen S, Isolauri E (2002). Probiotics: An overview of beneficial effects. Antonie Van Leeuwenhoek, 82: 279-289.

Pan X, Chen F, Wu T, Tang H, Zhao Z (2009). The acid, bile tolerance and antimicrobial property of *Lactobacillus acidophilus* NIT. Food Control, 20: 598-602.

Pekkanen J, Linn S, Heiss G, Suchindran CM, Leon A, Rifkind BM, Tyroler HA (1990). Ten-year mortality from cardiovascular disease in relation to cholesterol level among men with and without preexisting cardiovascular disease. Engl. J. Med., 322: 17-24.

Pennacchia C, Ercolini D, Blaiotta G, Pepe O, Mauriello G, Villani F (2004). Selection of *Lactobacillus* strains from fermented sausages for their potential use as probiotics. Meat Sci., 67: 309-317.

Pereira DIA, Gibson GR (2002). Cholesterol assimilation by lactic acid bacteria and bifidobacteria isolated from the human gut. Appl. Environ. Microbiol., 68: 4689-4693.

Rudel LL, Morris MD (1973). Determination of cholesterol using ophthalaldehyde. J. Lipid Res., 14: 364-366.

Saavedra L, Taranto MP, Sesma F, de-Valdez G (2003). Homemade traditional cheeses for the isolation of probiotic *Enterococcus faecium*

strains. Int. J. Food Microbiol., 88: 241-245.
Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: Cold Spring Harbor Laboratory Press Cold Spring Harbor, NY.
Schillinger U, Lucke FK (1989). Antibacterial activity of *Lactobacillus* sake isolated from meat. Appl. Environ. Microbiol., 55: 1901-1906.
Strompfová V, Lauková A, Ouwehand AC (2004). Selection of enterococci for potential canine probiotic additives. Vet. Microbiol., 100: 107-114.
Succi M, Tremonte P, Reale A, Sorrentino E, Grazia L, Pacifico S, Coppola R (2005). Bile salt and acid tolerance of *Lactobacillus rhamnosus* strains isolated from *Parmigiano Reggiano* cheese. FEMS Microbiol. Lett., 244: 129-137.
Tagg JR, Dajani AS, Wannamaker LW (1976). Bacteriocins of gram-positive bacteria. Microbiol. Mol. Biol. Rev., 40: 722-756.
Tamura K, Dudley J, Nei SK (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol., 24: 1596-1599.
Tannock GW (1999). Identification of lactobacilli and bifidobacteria. Curr. Issues Mol. Biol., 1: 53-64.
Tannock GW, Tilsala-Timisjarvi A, Rodtong S, Munro K, Alatossava T (1999). Identification of *Lactobacillus* isolates from the gastrointestinal tract, silage, and yoghurt by 16S-23S rRNA gene intergenic spacer region sequence comparisons. Appl. Environ. Microbiol., 65: 4264-4267.
Tuomola E, Crittenden R, Playne M, Isolauri E, Salminen S (2001). Quality assurance criteria for probiotic bacteria. Am. J. Clin. Nutr., 73: 393-401.
Upreti GC, Hinsdill RD (1973). Isolation and Characterization of a Bacteriocin from a Homofermentative Lactobacillus. Antimicrob. Agents Chemother., 4: 487-494.
Watanabe K, Fujimoto J, Sasamoto M, Dugersuren J, Tumursuh T, Demberel S (2008). Diversity of lactic acid bacteria and yeasts in Airag and Tarag, traditional fermented milk products of Mongolia. World J. Microbiol. Biotechnol., 24: 1313-1325.