International Journal of Sciences: Basic and Applied Research (IJSBAR)

ISSN 2307-4531
(Print & Online)

http://gssrr.org/index.php?journal=JournalOfBasicAndApplied

Health Benefit Characterization of Dominant Lactobacilli in Traditional Doogh

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Abstract

The aim of our study was the characterization and evaluation of isolated lactic acid bacteria(LAB) from traditionally produced Iranian Doogh and detection of the probable probiotic characteristics of the analyzed bacteria. We isolated 13 gram-positive and catalase-negative strains from traditionally produced Doogh and yoghurt. These strains and three control strains were investigated for anti-pathogenicity, hemolytic activity, antibiotic resistance, pH resistance and resistance against gastro enzymes in comparison to two established probiotic control strains. Identification was done by Biochemical API test and 16S rDNA sequencing methods. Eight strains had an observable inhibitory effect on bacillus cereus. No strains had hemolytic activities. 90% of isolates showed sensitivity to at least 7 of the 16 tested antibiotics, while just 15% had an intermediate sensitivity to Cloxacillin and Danofloxacin. 16 tested isolates were able to survive at pH 3 within 24 hours in phosphate buffer. 3 of the 16 Isolates suvived in simulated gastric condition for more than 2 hours at pH 2 and for 24 hours at pH 8. All identified strains were lactic acid bacteria belonging to *Lactobacillus spp* and one *P. acidlactici*. The composition of these strains might account for the specific sensorial and health benefits as well as stability of traditional Iranian Doogh.

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Keywords: lactic acid bacteria; Doogh; yoghurt; probiotics; hemolytic activity; antibiotic resistance; pH and bile resistance; 16S rDNA; API test.

1. Introduction

Fermented dairy products with their containing lactic acid bacteria play a main role in human diet all over the world. In addition to the function as starter cultures in fermentation, nonstarter lactic acid bacteria, especially probiotic bacteria with functional properties through metabolite production, increase the value of fermented food products [1]. Probiotic bacteria affect the composition of the human gastrointestinal (GI) microbiota and often contribute to improve dysbalances and inflammatory conditions [2]. Traditional and regional dairy products are an important reservoir of probiotic bacteria with health benefits. Some strains from traditional products demonstrate advantageous properties and tolerance against gastric enzymes and bile of GI tract [3, 4].

Doogh, a traditional fermented milk drink, is widely produced and consumed in tribes and villages of different regions of Iran [5]. It is produced from yoghurt dilution and shaking in leather stomach [6]. Bonyadi and his colleagues [7] enumerated lactic acid bacteria and yeasts in traditionally produced yoghurt samples from north east of Iran. It has been determined as 62×10^6 CFU/mL of lactobacilli in 1 ml and 41×10^4 CFU/ yeasts. In the study by Rashti et. al. 2015 [8], the analyzed microbial count of Doogh on MRS agar was about $2.2\text{-}6.8 \times 10^8$ in different kinds of produced Doogh from cow, sheep, goat and camel milk. In further studies from Azadnia et. al. 2009 more than 600 positive lactic acid cocci and bacilli were isolated and identified from traditional Doogh by cultivation and subjection to biochemical tests that *Leuconostoc mesenteroides subsp*. *cremoris* and *Lactobacillus delbrueckii subsp*. *bulgaricus* have been identified to be the dominant lactic acid bacteria [9]. Also probable probiotic bacteria like *lactobacillus plantarum* and *casei* have been isolated from fermented milk (Doogh) and other Iranian traditional dairy products [10]. The diversity of lactic acid bacteria in traditional dairy products differs by region and is not constant [6]. Diversity and characteristics of microbial content of traditionally produced Doogh and yoghurt may contribute to health in local Iranian communities through their probiotic bacteria content. Therefore, probiotic characterization of lactic acid bacteria of Doogh can be a possible way to identify health effects.

Main characteristics for selection and isolation of possible probiotic strains are acid and bile resistance factors. Other important criteria for probiotic strains are non-hemolytic activity, anti-pathogenicity, colonization, adhesion to mucosal surfaces and lack of antibiotic resistance gens that are commonly characterized by in vitro analysis [11].

Beside culture dependent methods, molecular methods including PCR and 16S rRNA gene sequencing as well as taxonomic identification by commercial kits like API 50 CH are increasingly used and improved the understanding of fermented products and their influences on GI microbiota [1, 12].

The purpose of our study was the characterization of heath promoting activities and identification of isolated lactic acid bacteria from traditional Iranian Doogh and yoghurt. We analyzed anti-pathogenicity, non-hemolytic activity, pH and enzyme resistance parameters using API 50 CH system and molecular identification. Selected

isolated strains from traditionally produced Doogh and yoghurt and probiotic control strains were used for identification analysis. All selected strains were gram positive with no catalase activity.

2. Materials and methods

2.1. Samples

20 sample of traditionally produced Doogh were collected from Kohkiloye and Boyer Ahmad and Hamedan provinces in Iran. Two regular yoghurts and one probiotic claimed yoghurt (containing bifidobacterium culture), commercially available, were applied as controls. From the 20 Doogh samples, 13 isolates and three isolated strain from control samples were used in our study (totally 16 strains).

2.2. Culturing and isolating

3 ml of the Doogh and yoghurt samples were inoculated in 50 ml MRS broth in falcon tubes sealed and incubated at 37^{o}C for 24 hour anaerobically. After centrifugation, the pellet was added to MRS broth with 0.3, 0.5 and 1 % bile (oxoid). After 48 hours anaerobic incubation at 37^{o}C, plated on MRS agar and re-incubated for 72 hours [4, 10]. As we were analysing probable probiotic lactic acid bacteria, for ensuring that the picked colonies are lactic acid bacteria they were checked for catalase activity according to [13]. After catalase test and gram staining, catalase negative and gram positive single colonies collected in TE buffer for molecular analysis and also stocked in MRS broth containing 40% (vol/vol) glycerol. We used 50 U nystatin (Sigma) / ml for inhibition of yeast and mold growing during cultivation [14].

2.3. Testing for antimicrobial activity against bacterial pathogens - agar diffusion method

The lactic acid bacteria isolates were tested for antagonistic activity against *Bacillus cereus* ATCC: 11778. This assay was performed in triplicates. An overnight culture of the pathogens in tryptone soya medium at 30°C for 24 hours and an overnight culture of the lactobacillus and streptococcus strains in MRS broth or in brain heart infusion at 37°C for 24 hours were prepared. Colonies from the cultures were fractionated and streaked on specific culture medium for the related pathogen bacteria; furthermore the cultures of lactic acid bacteria were filter-sterilized. With the cultivated colonies a turbidity equivalent of a 3.0 McFarland standard with a sterilized 0.85% NaCl solution was constructed. A lawn of an indicator strain was established by spreading the cell suspension with the McFarland of 3.0 over the surface of tryptone soya agar plates or COS agar plates (for *Streptococcus thermophilus*) with a cotton swab. The plates were dried, covered with sterile filter papers and 70 µl of filter-sterilized supernatant, obtained from culture grown in MRS broth, was pipetted onto filter papers. After incubation at 37°C for 24 hours, the diameter (mm) of the inhibition zone around the filter papers was measured [15].

2.4. Hemolytic activity

For testing of hemolytic activity, fresh lactic acid bacteria were streaked on Columbia agar plates (containing human blood) and incubated at 30°C for 48 hours. Clear zones around colonies are an indication for β-haemolysis, α-haemolysis are indicated with green zones, and γ-hamolysis appears with no zones around

colonies [16].

2.5. Antibiotic resistance

The antibiotic resistance was analysed by disc diffusion method. An overnight culture of the lactobacilli strains in MRS broth and streptococci strains in brain-heart infusion at 37°C for 24 hours was prepared. The cultures were fractionated streaked on MRS agar and plates were anaerobically incubated at 37°C for 24 hours. With these colonies a turbidity equivalent of a 0.5 McFarland standard with a sterilized 0.85% NaCl solution was prepared. Afterwards these cell suspensions were applied on the surface of MRS agar plates with a cotton swab and dried. Disks were then placed on the agar surface and anaerobically incubated at 37°C for 24 hours. After incubation the zone of inhibition was measured in millimetres [16, 17]. Resistance was defined according to the standard disc diffusion method on MRS agar by using 16 antibiotic discs. The zone of inhibition was divided into three groups: resistant, intermediate and sensitive.

2.6. Lactic acid bacteria in an acidic environment – resistance at a pH-value of 3

An overnight culture of lactic acid bacteria in MRS broth was prepared respectively in brain-heart infusion. With this culture a 2% dilution in MRS broth (pH 3) and brain heart broth (pH 3) was prepared. 0.5 ml of each solution was inoculated in 9.5 ml phosphate buffered saline (pH 3) and then the incubation mix was maintained anaerobically at 37°C for each time of investigation (0, 3, 6, 24 hours). Dilution series were prepared for each time of investigation in MRS broth in brain-heart infusion for streptococci. To determine total viable counts of lactic acid bacteria a pour plate method using MRS agar for lactobacilli and a spatula method on COS agar plates for streptococci, were used. The agar plates were incubated anaerobically at 37°C for 48 hours and viable colony counts were enumerated [4, 18].

2.7. Survival of lactic acid bacteria isolates under conditions simulating human GI tract – Transit tolerance

Preparation of simulated gastrointestinal juices: Simulated gastric juice was prepared by suspending 1.4 g of pepsin in 400 ml of 0.2 % saline and adjusting the pH to 2 with concentrated hydrochloric acid .Sterilization was accomplished by filtering through 0.45 µm filter. Simulated intestinal juice was prepared by suspending 0.4 g trypsin, 7.2 g bile salts, 4.4 g sodium bicarbonate and 0.8 g sodium chloride in 400 ml distilled water. The pH value was adjusted to 8 with sodium hydroxide; furthermore solution was sterilized by filtering through 0.45 µm filter.

Transit tolerance of selected strains: An overnight culture of lactic acid bacteria was prepared in MRS broth respectively in brain-heart infusion. With this culture a 1% dilution in MRS broth and brain-heart broth was constructed. Dilution was inoculated into simulated gastric juice at pH 2. The mixture was interspersed and incubated anaerobically at 37°C. Gastric transit tolerance was studied by determining total viable counts in gastric juice withdrawn at 0 and 3 hours. After 3 hours incubation in gastric juice, 1 ml culture was inoculated into 9 ml simulated intestinal juice (pH 8), and incubated anaerobically at 37°C for each time of investigation (0, 6, 24 hours). After incubation total viable counts in intestinal juice were determined [18, 19].

2.8. API 50CHL system

Selection of the colonies: To obtain cultures for analysis of carbohydrate degradation, the tested bacteria were first enriched in MRS broth and then cultured on MRS agar. The reference strains, which are collected as lyophilisate, were sub-cultured twice in MRS broth or brain-heart broth and subsequently isolated on MRS or COS agar plates.

Preparation of the strips: The preparation of the strips was done according to manufacturer's recommendation the API 50 CH kit.

Preparation of the inoculum: A suspension was prepared with a turbidity of 2 McFarland in the ampule of API 50 CHL medium with the pure cultures from the MRS- or COS agar plates. Inoculation of the strip: The tubes were filled with the inoculated API 50 CHL Medium, covered with paraffin and incubated aerobically at 37°C for 48 hours.

Reading and interpretation: After incubation, a positive acidification was indicated with Bromocresol purple (indicator), contained in the medium, by changing its color from red to yellow. Afterwards biochemical profile was identified using the api*web*^{TM} software [20].

2.9. Extraction of bacterial DNA from Doogh and yoghurt samples

DNA was extracted by centrifugation13000 rpm of Doogh samples; 500 mg of pellet was subjected to lysis and beat beating (30 sec, with one minute resting on ice) and then DNA was extracted using the QIAmp DNA Stool Mini Kit (Qiagen, Germany) following manufacturer's instructions. DNA was quantified and quality verified by Picodrop photometer measurement (Picodrop, UK) [21].

2.10. PCR and 16S rDNA gene sequence analysis

The 16S rDNA genes were amplified using primer set Lac1: AGCAGTaGGGaaTCTTCCA and Lac 2 ATTYCACCGCTACACATG with a ready-to-use GoTaq® Green Master Mix (Promega, USA). Primer concentration in total reaction volume was 0.5 pM and PCR has been done according to Zwielehner and Remely works in [22, 23]. PCR products were purified (QIAquick PCR Purification Kit) and tested on 2% agarose gel. 16 selected samples were sent for sequencing. Nucleotide sequences were analyzed with NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

3. Results

3.1. Antimicrobial activity against bacterial pathogens

In this study, the antimicrobial activities of supernatants were analysed at a concentration of 70 µl / discs. The data obtained from disc diffusion method indicated that 9 out of 16 tested isolated lactic acid bacteria strains, including three reference strains, showed an antimicrobial activity against the pathogen *Bacillus cereus* (Isolates 2, 5, 7, 8, 9, 10, 14, 15, 16) (Table 1).

Table 1: Antimicrobial activity against *Bacillus cereus* - agar diffusion method Reference strains: 9=*bifidobacteria*; 10=*Lactobacillus acidophilus*; 11=*Streptococcus thermophiles* (-) no zone, (+) 2mm< zone, (++) 2-4mm zone, (+++) 4-6mm zone of inhibition.

Strain Number	**Origin code**	**Inhibition zone**
1, 3 ,6, 11, 13	*YLS, DLS, DSB, RF3, YMG*	_
2, 5, 7, 8, 9, 10, 14,15	*DGG, DMK, DMV2, YSB, RF1, RF2, YLSA , DMV3,*	+++
16	DSBS	++

3.2. Hemolytic activity

Results showed that all examined strains did not exhibited α- and β-hemolytic activity when grown in Columbia human blood agar. All of the 16 tested strains were γ-hemolytic (none-haemolysis activity)

3.3. Antibiotic resistance

90% of the isolates, including reference bacteria, had a sensitivity to at least 7 of the 16 tested antibiotics, while none of them were sensitive to the veterinarian antibiotics Cloxacillin and Danofloxacin (Table 3).

3.4. Lactic acid bacteria in an acidic environment – resistance at a pH-value of 3.0

All of the tested isolates were able to survive at pH 3.0 during 3, 6 and 24 hours except the strain number 11 (Ref 3). Two strains showed very high resistance to low pH with final populations exceeding 5.54 or 3.97 log cfu/ml (Figure 1).

3.5. Survival of lactic acid bacteria isolates under conditions simulating human GI tract – Transit tolerance

In this test a great variation of growth among the test strains was observed in simulated gastric juice at pH 2.0. All strains showed a loss of viability during simulated gastric transit and almost all isolated strains suffered a complete loss of viability during simulated gastric passage. The three strains 9 (RF1), 14 (YLSA) and 15 (DM3) showed a significant level of survival in acidic and also alkaline pH of the simulated gastric juice and even kept their viability after 24 hours in simulated intestinal transit (Figure 2).

Table 2: Resistance behavior of lactic acid strains against 16 antibiotics. Reference strains: 9=bifidobacteria; 10=*Lactobacillus acidophilus*; 11=*Streptococcus thermophilus*

antibiotics	**resistant**	**intermediate**	**sensitive**
CFP (Cefoperazon)	1,2,4,5,6,7,8,10,12	13,14	3,9,11,15,16
CX (Cloxacillin)	1,2,3,4,5,6,7,8,9,10,11, 12, 13,14,15,16		
GEL (Ampicillin/Cloxacillin)		7,8	1,2,3,4,5,6,9,10,11, 12, 13,14,15,16
SYN (Amoxixillin/Clavulansäure)			1,2,3,4,5,6,7,8,9,10, 11,12, 13,14,15,16
PIR (Pirlimycin)	1,2,4,6,7,8,11	12,,13,14,15,16	3,5,9,10,11
ALB (Lincomycin/Neomycin)	3	3,5,7,13,14	1,2,4,6,8,9,10,11,12,15, 16,
DFX (Danofloxacin)	1,2,3,4,5,6,7,8,9,10,11, 12, 13, 14,15,16		
PEN (Penicillin)		1,2,4,6,7,8,13, ,16	3,5,9,10,11,12, 14,15
CFX (Cefalexin/Kanamycin)	1,2,3,4,5,6,7,8,10		9,11,12, 13,14,15,16
TS (Sulphamethoxazole/Trimethoprim)	3,5,7,8		1,2,4,6,9,10,11,12,13,14,15,16
TY (Tylosin)			1,2,3,4,5,6,7,8,9,10, 11,12, 13,14,15,16
MAR (Marbofloxacin)	1,2,3,4,6,7,8,9,10	11,14,15,162	5,12,13
RAX (Rifaximina)			1,3,4,5,6,7,8,9,10,11,12,13,14,15,16
AP (Ampicillin)	3,5,6,10,14	7,8,12,13	1,2,4,9,11,15,16
CEQ (Cefqzinom)	1,2,3,4,6,7,8,10,12, 13		5,9,11,14,15,16
MAL (Benestemycin/MamycinA/MamycinS)			1,2,3,4,5,6,7,8,9,10, 11,12,13,14,15,16

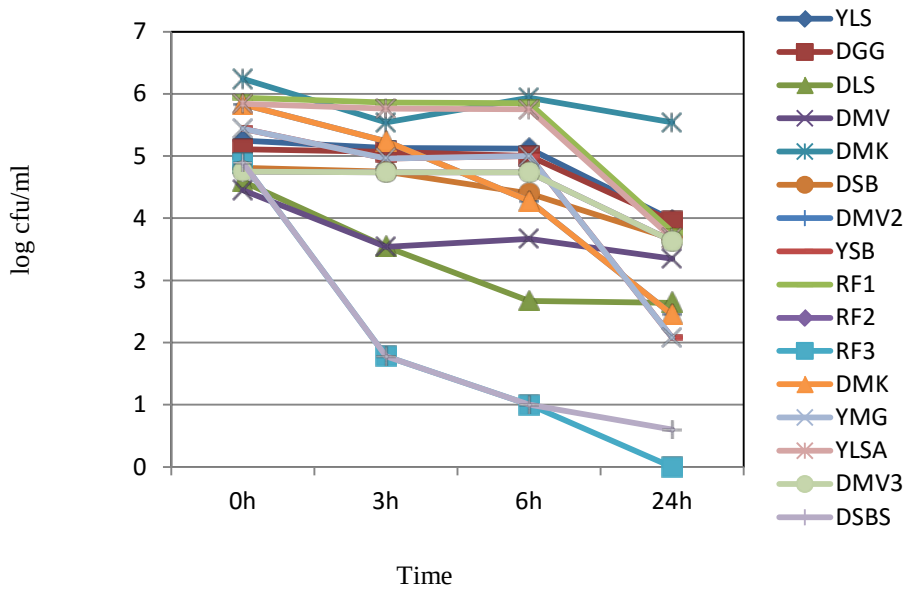

Figure 1: Survival rate of lactic acid bacteria in phosphate buffered saline (pH: 3.0)

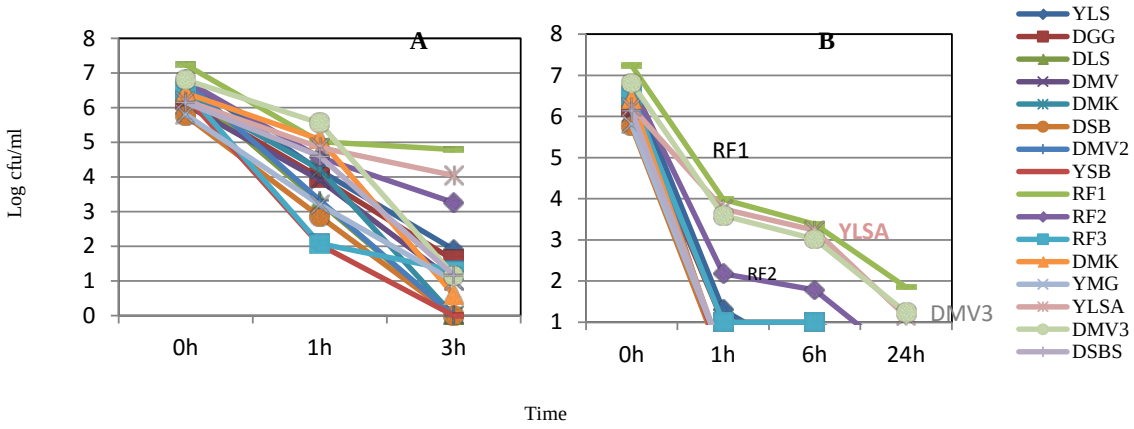

Figure 2: Survival rate of lactic acid bacteria strains after gastric transit in log cfu/ml at A; pH: 2 and B; pH=8

3.6. API 50 CH and 16S rDNA gene sequence analysis

The API 50 CH system was used for identification and discrimination of 13 isolated strains from dairy products. In addition 3 reference strains (*Lactobacillus acidophilus* and *Streptcoccus thermophilus*) were examined for their carbohydrate degradation.

Table 3 shows the results of identification by National Center for Biotechnology Information (NCBI) databases and the API 50 CH system. The results of the API 50 CH system for the strains 1, 2, 3, 4, 6, 8, 10, 12, 13, 14 and 16 are comparable to the results of the two databases.

Table3: 16S rDNA Sequencing and APT 50 CH results. Rreference strains: 9=bifidobacteria; 10=Lactobacillus acidophilus; 11=Streptococcus thermophilus

strain	**Origin code**	**via NCBI**	**Identity (%)**	**via API 50 CH**
1	*YLS*	*Lactobacillus brevis* **ATCC 367**	100	*Lactobacillus brevis 3*
2	*DGG*	*Lactobacillus brevis* **ATCC 367**	100	*Lactobacillus brevis 3*
3	*DLS*	*Lactobacillus buchneri* **NRRL B-30929**	99	*Lactobacillus buchneri*
4	*DMV*	*Lactobacillus brevis* **ATCC 367**		*Lactobacillus brevis 3*
5	*DMK*	*Lactobacillus buchneri* **NRRL B-30929**	98	invalid profile
6	DSB	*Lactobacillus brevis* **ATCC 367**	98	*Lactobacillus brevis 3*
7	DMV2	*Lactobacillus diolivorans*;	97	invalid profile
8	YSB	*Pedicoccus acidilactici* **B1104**	100	*Pediococcus acidilactici*
9	RF1	*Bifidobacterium*	100	invalid profile
10	RF2	*Lactobacillus acidophilus*	99	*Lactobacillus acidophilus*
11	RF3	*Streptococcus thermophilus*	98	doubtful profile
12	DMK	*Lactobacillus delbrueckii subsp. bulgaricus* **ATCC 11842**	100	*Lactobacillus delbrueckii ssp bulgaricus*
13	YMG	*Lactobacillus delbrueckii subsp. bulgaricus* **ATCC 11842**	100	*Lactobacillus delbrueckii ssp bulgaricus*
14	YLSA	*Lactobacillus acidophilus* **NCFM**	99	*Lactobacillus acidophilus*
15	DMV3	*Lactobacillus kefiranofaciens* **ZW3**	98	Invalid profile
16	DSBS	*Lactobacillus helveticus* **DPC 4571**	98	*Lactobacillus helveticus*

4. Discussions

We tested the isolated strains of traditional Doogh on effects on *bacillus cereus*, a gram-positive aerobic or facultative anaerobic bacterium. Toxins of this bacterium can cause vomiting and diarrhea in humans [24]. Nine isolates showed inhibition. More investigations with additional pathogenic strains are desirable. A study on anti-pathogenicity of traditional Doogh [25] showed that samples decrease the total Escherichia coli O157:H7 in a short time to less than 10 cfu/ml in comparison to industrial and probiotic strains. Therefore drinking traditional Doogh may contribute to eliminate the risk of bacterial food contamination.

Antibiotic resistance and transfer of resistance genes are present problems for probiotic strains. Decreasing

resistance, which can be transferred through food, is an active policy in European Union [26]. Due to potentially transferable antibiotic resistance genes, antibiotic sensitivity is a very important characteristic in selected strains as probiotics [27]. Our results of the examined lactic acid bacteria strains showed very different resistance to the 16 antibiotics. Reference control strains (9, 10, and 11) exhibited a low number of resistances. However, also Doogh derived strains 1 to 8, 13, 14 and 15 show low resistances to the most antibiotics we analysed.

According to WHO guidelines [11] for evaluation of strains from species with known hemolytic potential, determination of haemolytic activity is required. Normally most lactic acid bacteria do not have haemolytic activity and all our isolates showed no hemolytic activity consistently with other studies [28].

Being resistant to low pH is one of the major selection criteria for probiotic strains. To reach the small intestine they have to pass through stressful conditions of the stomach. The time during digestion in the stomach is about 3 hours. Acid resistance results show that Doogh derived strains show considerable resistance to acidic conditions. Tolerance of these strains to acidic environment was strain-specific in agreement to previous studies on lactobacilli strains resistance to low pH [18, 19].

Comparing the sequencing and API 50 CH system test shows very similar results in identification of lactic acid bacteria [29] but for differentiation between close *Lactobacillus* species these analytical systems may not be enough [30]. Molecular methods, such as sequencing of multiple rDNA regions, have to be used for specific strain characterizations.

Lactobacillus brevis and *Lactobacillus helveticus* have been isolated from traditional Doogh (Fars province) by Azadnia [9] with subsequent cultural and biochemical tests. *Lactobacillus acidophilus* isolation from traditional yoghurt from North West of Iran has been reported before [10]. *Pediococcus acidilactici* has been isolated by the authors in [6] from milk and Doogh (buttermilk) in Iran, which can survive high temperatures. Source of this strain can be the use of leather bags for Doogh production, or original regional used milk. *Lactobacillus acidophilus* NCFM is a known probiotic bacteria with distinct characteristics. It exists in conventional fermented foods and supplements, which are commercially available [31]. The strain number 15, identified as *Lactobacillus kefiranofaciens*, is mostly found in kefir and kefir grains. It has exopolysaccharide (EPS) production ability and therefore may have emulsifying capability, with indirect probiotic activity [32, 33].

The average amount of dairy product consumption in Iran is equal to 139 g per day or at least 10 % (w/w) of food basket that for urban area is 142 g and for villages is 134 g. Yoghurt, cheese and milk are the most consumed dairy products [34]. Unfortunately, the official amount of Doogh consumption is not available but estimations are 28.5 kg per year, or 78 g per day [35]. It seems Doogh and yoghurt consumption in tribes and villages of Iran underlie seasonal changes and are therefore higher in milk production periods. The claimed amounts for yoghurt consumption could be including the amount of Doogh consumption.

One important point about traditionally produced Doogh is that it is prepared with unknown starter cultures (unknown characteristics for health and quality texture, flavor and appearance). Additionally bacteria come from added water; leather bags, dishes etc. play a role in fermentation. Microbial community of Doogh may

also vary locally.

5. Conclusion

This study shows that traditional Doogh is a valuable source for health promoting bacteria that could tolerate GI tract conditions (bile and acid resistance for the strains). These bacteria can in future be used for industrial purposes i.e. development of new probiotic dairy products. More studies on microbial diversity of Doogh production by using leather bag (Mashk) and clinical studies on health consequences are necessary. The role of traditional Doogh on health and GI microbiota, and the intervention of consuming traditional fermented dairy products should be investigated in different regions of Iran.

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