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Phenotypic and Genotypic Characterization of Lactic Acid Bacteria from Traditional Cheese in Khorramabad City of Iran with Probiotic Potential

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Abstract Lactic acid bacteria (LAB) with proteolitic activity are used as aromatic and antibacterial substances, cholesterol reduces, bile salt hydrolyses, and probiotic. The aims of this project were to isolate and identify natural LAB flora involved in traditional fermentation in cheeses of Khoramabad city and also to survey their probiotic potential. In order to achieve this goal, LAB were isolated and characterized using phenotypic and genotypic methods (PCR-sequencing); in the next stage, they were analyzed lowering cholesterol medium, hydrolysis of the bile, resistance to bile-resistant PH acidic stomach. At the end of the study, 88 cocci and 3 bacill were found: *58 Enterococcus faecium*, 16 *Enterococcus hirae*, 5 *Lactococcus lactis*, 3 *Lactobacillus plantarum*, and 9 undetermined. The probiotic results of the bacteria had effects on the reduction of cholesterol, resistance to stomach acid, had relative antibacterial effects, and some strains had effects on hydrolyzing the bile. For further identification, the PCR method and the application of 16s-DNA-ITS genes and its sequencing were found useful. This study showed that lactic acid bacteria in the traditional cheese of the Khorramabad city have relative probiotic effect and that these lactic acid bacteria in fermented milk are suitable.

Keywords Lactic acid bacteria · Probiotic · PCR · Sequencing

Abbreviations

CFU	Colony-forming units
dH_2O	Deionized water
LAB	Lactic acid bacteria

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MRS	deMan-Rogosa-Sharpe
MRSBA	deMan-Rogosa-Sharpe-Bile salt-Agar
MRSCHO	deMan-Rogosa-Sharpe-CHolesterol Oxgall
MRSO	deMan-Rogosa-Sharpe-bile Oxgall
MRSV	deMan-Rogosa-Sharpe-Vancomycin

Introduction

The production of cheese from milk is an ancient process. Cheese manufacturing started about 8000 years ago in the Fertile Crescent, between Tigris and Euphrates rivers. Production of cheese is essentially achieved by bringing four ingredients together: milk, rennet, microorganisms, and salt. This process has the following stages: formation of mass, acid production, the rejection of the cheese water, addition of salt, and finally cheese processing. The important biochemical changes taking place in cheese production are the production of lactic acid from lactose which occurs through different types of lactic acid bacteria. Floras causing the progress in the production of acid during the production of cheese are used as starter cultures which reduce pH and the formation of mass and rejection of water. In the early years of the first decade of 1900s, there has been a remarkable increase in the industrial production of cheese. Due to the importance given to hygiene being one of the major criterions in large-scale production, for this reason, pasteurized milk is used. Therefore, natural lactic acid bacteria (LAB) flora contained in the milk is lost. Consequently, in order to make cheese from pasteurized milk, an external LAB source is needed. This source includes predefined strains of LAB which are called starter strains. In Turkey, there has been no starter strain developed as yet that could represent our native LAB flora. This prompted us to collect LAB from the regions where industrial cheese making is still dominated. Traditionally, milk from cow, goat, and sheep is fermented by the help of naturally occurring indigenous LAB. Alongside the technological parameters like curd handling and cooking temperature, the quality of cheese is mainly dependent on the microbial associations within the respective region. Therefore, the LAB flora of traditional cheese making can be taken as the basis of starter strains with unique characteristics. In other words, to prevent the loss of microbial diversity and loss of wide range of cheese variety, building up LAB collections is a very important task. Having the traditional production and consumption of dairy products including cheese within the province of Lorestan, it is important that the LAB I these products be identified and also the probiotic potential for healthy consumption and better use and their commercial advertisement be identified. Considering that cheese industries across the plant are of the biggest fermentation industries, special attention is needed to be paid to them. According to research done in the USA from 75 billion milk products in 2001, more than a third of it was used in the production of 3.7 billion kg of cheese. Also, the annual consumption of cheese in the USA in the same year was 8.198 kg, which was enhanced to 14 kg in 2003 which shows that attention must be paid to this industry for public health control.

One of the most important criteria for selection of probiotic organisms is their ability to survive in the acidic environment of the product and in the stomach, where the pH can reach as low as 1.5. Similarly, the organisms must be able to survive in the bile concentrations encountered in the intestine. Studies [1] showed that, among several strains of *Lactobacillus acidophilus* and *Bifidobacterium* sp., only a few strains survived under the

acidic conditions and bile concentrations normally encountered in fermented products and in the gastrointestinal tract, respectively. Therefore, it cannot be generalized that all probiotic strains are acid and bile tolerant.

The selection of potential probiotic strains that would be capable of performing effectively in the gastrointestinal tract is a significant challenge. Bile is a yellow-green aqueous solution whose major constituents include bile acids, cholesterol, phospholipids, and the pigment biliverdin. It is synthesized in the pericentral hepatocytes of the liver, stored and concentrated in the gallbladder interdigestively, and released into the duodenum after food intake. Bile functions as a biological detergent that emulsifies and solubilizes lipids, thereby playing an essential role in fat digestion. This detergent property of bile also confers potent antimicrobial activity, primarily through the dissolution of bacterial membranes. The primary bile acids, cholic and chenodeoxycholic acid, are synthesized de novo in the liver from cholesterol. The solubility of the hydrophobic steroid nucleus is increased by conjugation as an *N*-acyl amidate with either glycine (glycoconjugated) or taurine (tauroconjugated) prior to secretion. The resulting molecules are therefore amphipathic and can solubilize lipids to form mixed micelles.

Experiments [2] have shown that dietary elevation of plasma cholesterol levels in pigs can be prevented by introduction of a *Lb. acidophilus* strain that is bile resistant and assimilates cholesterol. These findings were supported by research conducted [3] and demonstrated that probiotic strains were able to assimilate cholesterol in the presence of bile into their cellular membranes. Results, however, were influenced greatly by the bacterial growth stage and inoculum used as resting cells did not interact with cholesterol. Given that dairy products and the ability of probiotic bacteria have not been studied so far, in Khorramabad city of Lorestan province, the type of probiotic bacteria and their potential in our study has been questioned. Finally, to assess and interpret the results, SPSS software was used.

Intestinal microflora is a complex ecosystem. Introducing new organisms into this highly competitive environment is difficult. Thus, organisms that can produce a product or products that will inhibit the growth or kill existing organisms in the intestinal milieu have a distinct advantage. The growth media filtrates and sonicates from the bacterial cells of prospective probiotics should be tested for bactericidal and bacteriostatic activity in well plates against a wide variety of pathogens. The ability of probiotics to establish in the gastrointestinal tract will be enhanced by their ability to eliminate competitors.

For that, bacteria known as probiotic must have no virulence factor or resistant gene. That is why resistant gene with antibiogram method that must be tested would help in stepping up the public health. We hope the results of this research take a step in the improvement of public health.

Materials and Methods

In this study, 13 types of cheese prepared from fresh cow milk were collected from different entrances in Khorramabad city. The majority of them were made of fresh cheese, and others were ripen cheeses. Samples were analyzed by the dilution pour plate method. For this purpose, 10 g of each sample was weighed aseptically and homogenized in 90 ml of sterile trisodium citrate 2 % solutions in a handmade stomacher. Then, sequential decimal dilutions of the homogenate were obtained. One-milliliter aliquot of the 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} dilutions was used for the isolation of LAB. Based on the culture media and conditions (pH and incubation temperatures), every sample was plated into these selective media.

MRS agar (pH 6.2–6.8): Isolation and enumeration of *Lactobacilli*
M17 agar (pH 7.2): Isolation and enumeration of *Streptecocci* and *Lactococci*
MRSV agar (pH 7.2): Isolation and enumeration of *Leuconostoc*
Glucose yeast extract agar (pH 7.2): Isolation and enumeration of *Pediococcus*

All plates contained 0.3 % salt for securing in comparison to bile salt. MRS plates were incubated under microaerophilic conditions using gas generating kit (Oxoid). All plates were incubated for 3 days at 37 °C. After the incubation, the plates with CFU ranging from 30 and 300 were selected for enumeration. Isolates showing homofermentative, Gram-positive, and catalase-negative characteristics were preserved in MRS broth medium which contained 25 % (*v*/*v*) gycerol as frozen stocks at −80 °C.

Physiological and Biochemical Identification

Identification of Isolate

Each isolate was activated in 5-ml MRS broth for 24 h at 30 °C before use. Then, overnight cultures were used during all the identification procedures. Physiological and biochemical identifications were performed according to the methods and criteria [4–6]. The characteristic used for the identification of different shapes of cocci LAB was presented in this study.

For all tests, a negative control (noninoculated media) was also used, and respective reference strains were also included in the experiments as positive controls.

Reference strains used in the study as fallows:

1. *Streptococcus faecium* (*Entrococcus hiraie*) *PTCC 1238*
2. *Lactobacillus acidophilus PTCC 1643*

Gas Production from Glucose

In order to specify the homofermentative samples, the co2 test of glucose was carried out. For this purpose, citrate lacking MRS broths and inverted Durham tubes were used. A total of 50 μl of overnight cultures was transferred into the 8-ml test media. After incubation for 3 days at 37 °C, gas production from glucose was surveyed.

Growth at Different Temperatures

A total of 50 μl of overnight cultures was transferred into the 5-ml temperature test media containing bromococozal propel. After inoculation, they were incubated for 5 days at 10, 40, or 45 °C. Cell growth at any of these temperatures was detected by the change in the color of the cultures, from purple to yellow.

Growth at Different NaCl Concentrations

A total of 50 μl of overnight cultures was transferred into the tubes which contained 5 ml of NaCl test media. Isolates were tested for growth at 2, 4, or 6.5 % NaCl concentrations. They were incubated for 5 days at 37 °C. The changes of the color from purple to yellow were taken as the evidence for cell growth.

Arginine Hydrolysis and Gas Production from Citrate

In order to perform this test, 5 ml of Reddy broth and inverted Durham tubes were used. A total of 50 μl of overnight cultures were inoculated into the Reddy broth and were then incubated for 5 days at 37 °C.

Arginine Hydrolysis The cultures, which utilize arginine, change the color of the broth first to yellow due to the lactic acid production and then to violet because of the ammonia production. On the other hand, the cultures which do not utilize arginine assume a deep-yellow color by producing lactic acid only.

Fermentation of Carbohydrates

Isolates were characterized on the basis of their sugar fermentation profiles. All the reactions were performed by using 96-well microtiter plates [7]. Thirteen different sugars were used. For each test, strains were inoculated in 5-ml MRS broth (50 ml/l) and were then incubated for 24 h at 37 °C in order to obtain overnight cultures. After this, cultures were centrifuged for 10 min at 10,000 rpm. Pelleted cells were washed and resuspended in MRS (without glucose) containing bromocresol purple as the PH indicator. A total of 20 μl of filtered and sterilized (0, 22 μm, Millipore) 10 % sugar solutions were pipetted into each well. On to the sugar solutions, 180 μl of suspended cells was added. Thus, 1 % final sugar concentration was obtained. Duplicate reactions were prepared for each of the sugar fermentation experiments. After 24-h incubation at 37 °C, when the sugar fermentation was taken place, the color changed from purple to yellow, and turbidity was increased. Glucose fermentation included to positive control, and samples without sugar were used as negative control.

Methods Survey Probiotic Potential

Bile Tolerance

Our isolates were incubated in MRS broth at 37 °C for 16 h. Then, we harvested the cells by centrifugation at 5000 rpm for 20 min. Washed twice the pellet obtained with 0.1 M phosphate buffer, pH 7.0. Resuspend the cells to the original volume with the buffer by vortexing. Inoculate (0.5 %) MRS and MRSO broth with the bacterial suspension. Incubated at 37 °C in bath water. Optical density at 560 nm (OD560) against the blank (uninoculated broth) every hour for the first 8 h and after 24 h of incubation was read. Plot optical density values against incubation time.

Resistance to Low PH

Grew the isolated under study in MRS broth at 37 °C for 16 h. Harvested the cells by centrifugation at 5000 rpm for 20 min. Washed twice the pellet obtained with 0.1 M phosphate buffer, pH 7.0. Resuspend the cells to the original volume with the buffer by vortexing. Inoculate (2 %) the artificial gastric juice pH 2–2.3 and pH 6.5–7.0 with the bacterial suspension. Incubated both media at 37 °C and take samples at 0, 1, 2, 3, and 4 h and after 24 h for cell viability.

Plate in MRS agar (in mass) proper dilutions from 10-fold serial dilutions prepared in 0.1 % peptone water. Incubated the plates at 37 °C for 24 h, and counted the resulting colonies after that time. Results are expressed as colony-forming units (CFU) per milliliter (CFU/ml).

Cholesterol Reduction

Inoculated (1 %) 20 ml of MRSCHO broth and overnight culture in MRS broth and incubated at 37 °C for 16 h. Uninoculated MRSCHO broth (control) is processed in the same way. Remove the cells by centrifugation at 8000 rpm for 5 min. Place the sample (0.5-ml supernatant) into a clean glass tube. Add 3 ml of 95 % ethanol to each tube, followed by 2 ml of 33 % potassium hydroxide. Mix after the addition of each component.

Heat the tubes in water bath at 60 °C for 10 min. Cool at room temperature (20 °C). Carefully added 5 ml of hexane. Mix vigorously with a vortex for 20 s. Add 3 ml dH2O and repeat the mixing with the vortex. Let the tubes settle at room temperature for 15 min until separation phase is complete (aqueous and organic phase). Transfer 2.5 ml of the hexane layer (upper phase) into a clean tube. Evaporate hexane to dryness at 60 °C under nitrogen gas flow. Resuspend the residue formed in 4 ml of *o*-phthalaldehyde reagent. Keep the tubes at room temperature for 10 min and then pipet 2 ml of concentrated sulfuric acid slowly down the inside of each tube. Mix thoroughly as described previously. After standing at room temperature for an additional 10 min, read the absorbance at 550 nm (A550) against the reagent blank. The results are expressed as micrograms (μg) of cholesterol per milliliter.

Bile Salt Hydrolase Activity

Melt the agar media which include MRS agar and MRSBA agar in boiling water. Pour each melted medium separately into sterile Petri dishes (60×15 mm). Once solidified, invert the plates and place in an anaerobic chamber for at least 48 h before using. Inoculate each plate on surface with an overnight culture grown in MRS broth by using a 10-μl loop. Incubate the plates at 37 °C in anaerobic jars (Systen Oxoid) for 72 h. The bile salt hydrolase activity of the cultures is evidenced by the formation of a white precipitate around the colonies grown in MRSBA agar. This precipitate is not observed in MRS agar (control) without bile salts, where colonies are translucent. [8]

Antibacterial Assay

Agar well diffusion method [9] was used to detect antimicrobial activities of CFSs produced from isolated bacteria. After the preparation of pure culture in MRS agar, 5 ml of the culture medium was prepared in MRS broth, and bacterial cells were removed by centrifuging the culture at 10,000 rpm for 5 min, and the supernatant was filtered using 0.22-μm membrane filter. Paper disks were treated in a CFS solution. On the other hand, indicator strains such as *Pseudomonas aeruginosa* (PTCC 1430), *Listeria monocytogenes* (ATCC 345), *Proteus vulgaris* (ATCC 1312), *Staphylococcus aureus* (ATCC 64542), *E. coli* (ATCC 2143), *Bacillus cereus* (ATCC 1015), and *Bacillus subtilis* (ATCC 1156) and *Sterptococcus feacalis* (PTCC 1237) were inoculated in the TSB (0.5 on the McFarland scale). Then, indicator strains were swapped with sterile culture in Muller Hinton Agar (MHA). Then, paper disk was dipped into the medium and was placed in the plates with gaps between them and the walls. And then, inhibition zone was measured after incubation incubated for 24 h at 37 °C.

Determination of Antibiotic Resistance

Disk diffusion was used for determining antibiotic activity. Antibiotic disks that were used include the following: trimethoprim (15 μg), amoxicillin (10 μg), tetracycline (30 μg) erythromycin (15 μg), kanamycin (30 μg), clindamycin (2 μg), rifampin (5 μg), and vancomycin (30 μg).

Each of them was put on an MRS agar with a distinct gap between each other. Inhibition zone was measured accurately, and then, the results were analyzed using SPSS software.

DNA Extraction, Amplification, and Denaturing

Gradient Gel Electrophoresis Genomic DNA from pure cultures was extracted with the bioneer kit (korea). The PCR amplification of approximately 2000 bp of the ITS region of the 16S rDNA gene was obtained using the following:

Forward primer EGE1: 5′-AGAGTTTTGATCCTGGCTCAG-3′ (Mora et al. 1998) [10]
Reverse primer L1: 5′-CAAGGCATCCACCGT-3′ (Jensen et al. 1993) [11]

This PCR reaction was performed in total reaction volume of 25 μl containing 2.5 μl of 10× PCR buffer, 0.5 μl of each of the primers, 0.75 μl of $MgCl_2$, 0.5 μl of dNTP 1 U of Taq DNA polymerase, and 3 μl of the sample. The amplification program was as follows: 94 °C for 5 min, 40 cycles of 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min, and then 72 °C for 10 min. The PCR product for sequencing was sent to Bioneer Co. in Seoul, South Korea.

Results

LABs were initially grown in agar plates containing MRS (pH 6.2–6.8), M17 (pH 7.15), MRSV (pH 7.2) media, and glucose yeast extract agar (pH 7.2) for 48 h and 37 °C; then, the colonies were counted. The mean of the counted LAB in new samples of MRS, M17, MRSV, and glucose yeast extract agar were more than the ripen cheese samples. The high concentration of Na Cl in the ripen cheese had a negative effect n LAB and caused their growth to cheese. Additionally, this study showed that the mean of log CFU/g of the LABs during milk clotting increased and in ripen cheese decreased a little. Therefore, the research was carried out on fresh and clotted cheese.

From 13 cheese samples, the following results were obtained.

Morphological Examination

Isolates were analyzed under the light microscope. At this step, cell shape (like cocci, ovoid, rod) and their arrangements (like diplo form, chains form, tetrad form) were examined after simple staining. Thirty of the isolates showed positive catalase and were eliminated. Finally, 91 pure isolates which include 88 of cocci and 3 of bacilli were obtained.

Physiological and Biochemical Tests

In order to identify the shape, isolated were used from bergey table. The isolates that were able to metabolize citrate were listed. Sugar fermentation profiles provided identification at species

level for our typical Enterecoccal isolates. Arabinose, sorbitol, and glycerol provided discrimination between *E. faecium* and *E. faecalis*. At the end of the study, a total of 88 cocci and 3 lactobacillus were isolated. It was found that cocci and bacill-shaped isolates included *58 E. faecium*, 16 *E. hirae* , 5 *L. lactis*, 3 *Lb. plantarum*, and 9 were not determined.

Test Result Probiotic Potential

Resistant to Low pH

Being resistant to low pH is one of the major selection criteria for probiotic strains. Since, in the process of reaching the small intestine, they have to pass through from the stressful conditions of the stomach. According to this experiment, five isolates were resistant to low pH. Two selecting strains resistant strains in low pH 2.5 and pH 7. All results were recorded and analyzed with SPSS softward (Fig. 1).

Five isolated include *Lb. plantarum* (GB2), *L. lactis* (EC55), *E. faecium* (DC67), *E. faecium* (KC100), *E. hirae*(GC93), had appropriate resistant for low pH and selected for doing the following tests.

Tolerance Against Bile

Isolated with resistant to low pH were assayed to tolerate bile salt. Although the bile concentration of the human gastrointestinal tract varies, the mean intestinal bile concentration is believed to be 0.3 % *w*/*v*, and the resistance time is suggested to be 4 h. Strains were detected in 0.3 % during 4 h. The CFU values and OD620 in MRS and MRSO broth were observed.

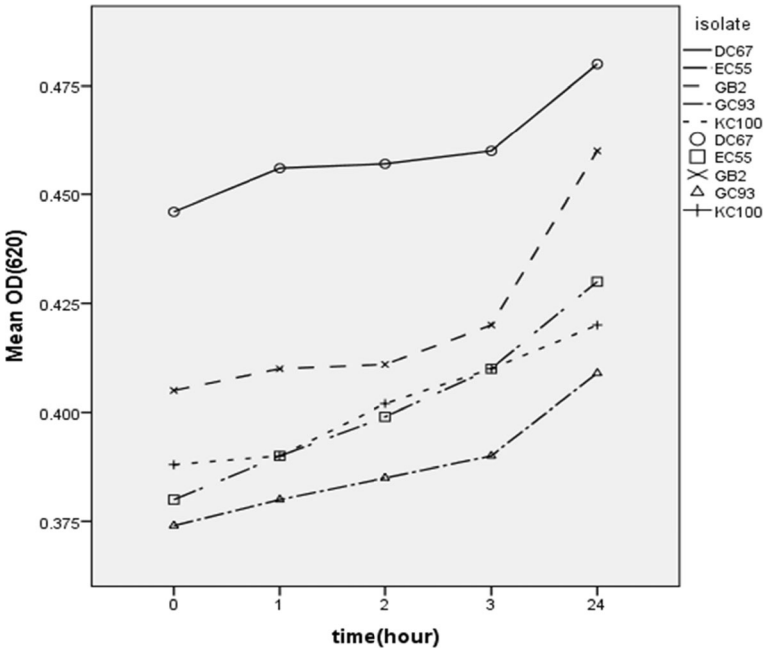

Fig. 1 Survival in pH 2.5 CFU values

According to the results, all of the isolates are resistant to 0.15 % bile salt, and most of them were resistant to 0.3 % bile salt.

The difference in increased time (min) compared to the time required for each of them to increase by 0.3 units in MRS broth medium containing 0.3 %, and no gall bladder for 4 identified strain was resistant to acid and delays.

(D) were expressed in the following minutes (Table 1).

Very resistant with growth delay time from 0 to 30 min.

- Resistant with growth delay time from 30 to 60 min.
- Tolerant with growth delay time from 60 to 90 min.
- Sensitive with growth delay time from 90 and 120 min.
- Very sensitive with growth delay time from 120 to 180 min.

Bile Salt Hydrolysis Activity

In this research, catalyze activity enzyme BSH and hydrolyse salts were assayed, and the hydrolysis of bile salts after cultivation on the MRSBA agar showed that strains of EC55, GC93, KC100, GB2 which had effected hydrolysis of the bile salt, and DC67 had no effect on the hydrolysis of the bile salt.

Cholesterol Lowering

The percentage of cholesterol reduction in the isolated after 24 h at 37 °C showed that this isolated can reduce chlesterol. Results after 24 h at in vitro condition showed that *E. hirae* (GC93) 50.5 %, *E. faecium* (DC67) 31 %, *L. lactis* (EC55) 52 %, *E. faecium* (KC100) 52.6 %, *Lb. plantarum* (GB2) 28.1 % reduced cholesterol.

Antibacterial Assay and Antibiotic Resistance

The bacterial activities exhibited by our isolated are presented in Table 2. Antibacterial activity against seven types of pathogens was evaluated. Results showed maximum diameter of inhibition zone for *Lb. plantarum* isolated against *Staphylococcus aureus* (14 mm) and minimum inhibition zone against *Bacillus cereus* and *E. coli* that was resistant to them.

Most of the isolates had good resistance to all of the antibiotic disks used in this study. Results concerning the determination of antibiotic resistance of the isolates are given in Table 3.

Table 1 Resistance to bile salt

Resistance to bile	Growth delay (min)	Isolate code
Very resistant	30>	GB2
Very resistant	30>	EC55
Very resistant	30>	DC67
Sensitive	90>X>120	GC93
resistant	30>X>60	KC100

Table 2 Activity spectrum of cell-free supernatants of *Enterococcus faecium* and *Lactococcuslactis* against several pathogenic strains

	Inhibition zone	
Indicator strains	Enterococcus faecium	lactococcuslactis
1- *Pseudomonas aeruginosa*	8.0±0.5	8.0±0.3
2- *Staphylococcus aureus*	12.0±0.7	12.0±0.5
3- *E.coli*	–	–
4- *Bacillus cereus*	–	–
5- *Bacillus subtulis*	8.0±0.3	7.0±0.5
6- *Proteus vulgaris*	*10.0±0.4*	*10.0±0.0*
7-sterptococcus faecalis	*10.0±0.5*	*10.0±0.5*
Listeria monocytogenes	*7.0+0.0*	*8.0+0.2*

PCR-Sequencing

All isolates were preliminarily identified by phenotypic methods, and the most promising isolates were identified by 16S rDNA gene sequencing. In order to identify LAB by PCR-sequencing technique, 16S rDNA were amplified. The length of amplification products was 2000 bp (Fig. 2).

Table 3 Antibiotic resistant isolated

Antibiotic	VAN	KAN	AMO	TTC	RIF	ERY	CC	SXT
Resistance of Enterococci faecium DC67 to antibiotic								
resistant		R			R			R
Intermediate				I			I	
sensitive	S		S			S		
Resistance of Enterococci faecium KC100 to antibiotics								
resistant		R		R		R	R	R
Intermediate								
sensitive	S		S		S			
Resistance of lactococcus lactis EC55 to antibiotics								
resistant	R						R	
Intermediate		I						
sensitive			S	S	S	S		S
Resistance of Enterococci hirae GC93 to antibiotics								
resistant		R			R	R		R
Intermediate								
sensitive	S		S	S			S	
Resistance of Lactobacillus plantarum GB2 to antibiotics								
Resistant								R
Intermediate								
Sensitive	S	S	S	S	S	S	S	

VAN vancomycin, *AMP* ampicillin, *KAN* kanamycin, *RIF* rifampicin, *CC* chloramphenicol, *ERY* erythromycin, *SXT* sulfamethoxazole-trimethoprim, *TTC* tetracycline, *R* resistant, *I* intermediate, *S* sensitive

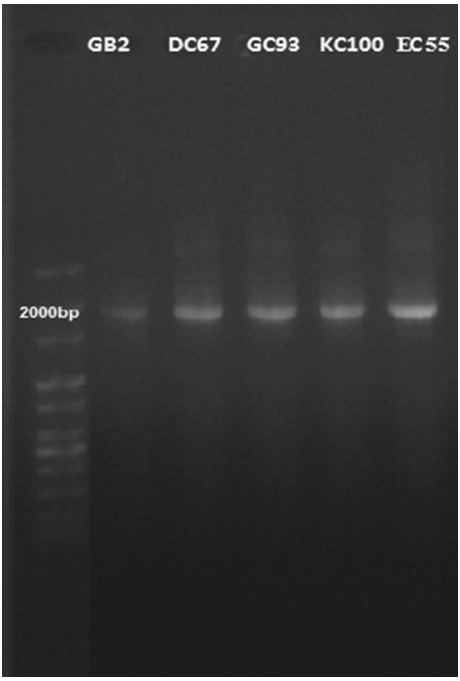

Fig. 2 Represantative 16 SrDNA amplification products of isolates

Our results demonstrate for five isolated from cheese. Similar observations have been reported with phenotypic. Results are given in Table 4.

Discussion

In this study, a total of 98 cocci and 3 lactobacillus were isolated. It was found that cocci and bacill-shaped isolates included 58 *E. faecium*, 16 *E. hirae*, 5 *L. lactis*, 3 *Lb. plantarum*, and 9 were not determined. The effect of probiotic bacteria identified in this study was to prove that all isolate were resistant to acids, isolate *Lb. plantarum* GB2, *L. lactis* EC55, and *E. faecium* DC67 had most resistance against bile salt, all isolates had hydrolyzing effects on bile salt except *E. faecium* DC67, all had the effect of lowering cholesterol within the range of 25 to 55 % of the maximum for *Lb. plantarum* GB2 that was55% and *E. faecium* DC67 50 %.

Table 4 Result of 16S rDNA sequencing of isolates

Isolate	Species	Similarities (%)	Accession number
GB2	*Lactobacillus plantarum* subsp. plantarum ST-III	99	NC_014554.1
EC55	*Lactococcuslactis* subsp. cremoris UC509.9 chromosome	96	NC_019435.1
DC67	*Enterococcus faecium* DO chromosome	99	NC_017960.1
GC93	*Enterococcus hirae* ATCC 9790 chromosome	99	NC_018081.1
KC100	*Enterococcus faecium* DO chromosome	99	NC_017960.1

This study showed that *E. faecium* and *L. lactis* had an inhibitory effect on pathogen bacteria and improved infection or prevented infection in the body, these bacteria also had the appropriate antibiotic resistance against most antibiotics that due to having this benefit effects could serve as an adjuvant, added to food specially dairy products consumed and increase safety against pathogenic bacteria.

The isolation of LAB due to their special qualities has major importance in industry and science. In the future, the following characteristics for isolates would be useful.

1. Anticarcinogenic effects
2. Nonspecific response to the immune system
3. Effect of increased calcium absorption, which leads to inhibition of bone decalcification in the senility.
4. Synthesis of vitamins.
5. Bacteriophage resistance.

Results obtained in this study confirmed that strain isolation from traditional cheese of Khorramabad city, Iran, could serve as a useful alternative and be useful for previous nonstarter that could be of assistance to the dairy industry in the future and with this study steps to be taken to improve public health.

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