Identification and Characterization of *Lactococcus lactis ssp.lactis* Isolated from Raw Milk and Milk Products of Turkey and Iran

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ABSTRACT— ***Lactococcus lactis ssp.lactis strains are the best known and characterized species of lactic acid bacteria which plays an important role as starter cultures in industrial dairy fermentation, owing to its pH lowering effect, production of antimicrobial metabolites and enhancement of the organoleptic attributes. The objective of this study was to identify and evaluate the varied properties of the natural L.lactis ssp.lactis strains from raw milk and dairy products obtained from different provinces of Turkey and Iran during the period of April to June 2016. Out of 262 dairy samples, L.lactis ssp.lactis were isolated from 98 samples. In order to evaluate the obtained strain as a good quality starter culture, litmus milk reaction, total titratable acidity, skim milk agar, antimicrobial activity and antibiotic resistance tests were conducted. The genetic basis of the observed phenotypic resistance obtained by disc diffusion test was further confirmed by PCR. Lactic acid production by Iranian cheese samples were significantly higher than Turkish samples (p<0.05) although difference between their proteolytic activities were statistically insignificant (p>0.05). Iranian cheese isolates showed higher antimicrobial activity againts E. faecalis and E. coli as compared to Turkish isolates. (p<0.05). Difference in antibiotic resistance between Turkish and Iranian cheese samples were not statistically significant (p>0.05). However, tetracycline and streptomycin resistance gene was only confirmed by PCR in Turkish cheese samples. Emphasis must be given to selection of the milk source for obtaining good quality starter cultures. Further studies may be conducted to learn the effect of ecological parameters on bacterial strains.***

Keywords— Lactic acid bacteria, *L.lactis ssp.lactis*, starter culture, dairy products, antimicrobial, antibiotic

1. INTRODUCTION

Lactococcus lactis strains (*L.lactis* ssp.*lactis*, *L.lactis* ssp.*cremoris* and *L.lactis* ssp. *lactis* biovar.*diacetylactis*) are the best known and characterized species of lactic acid bacteria (LAB) which are mainly isolated from either dairy product or plant material [32]. LAB plays an important role as starter cultures in industrial dairy fermentation. Owing to its pH lowering effect and production of antimicrobial metabolites it is used in the food industry as an additive enhancing the organoleptic attributes, shelf life and product quality [19, 24, 43]. *Lactococcus* species are of special importance in ripening of cheese matrix due to its ability to produce intracellular peptidase enzymes.

Selected *L.lactis ssp.lactis* strains were suggested to have health promoting effects. Some strains favourably influenced the intestinal flora of human and animal hosts, led to competitive exclusion of gastrointestinal pathogens, stimulated immune responses and exhibited anti-carcinogenic properties [27, 28].

Dairy products containing *L.lactis ssp.lactis* strains were found to improve lactose digestion and absorption in lactose intolerant individuals [10] as well as reduce heart rate and blood pressures in hypertensive rats, beneficial in the prevention and treatment of cardiovascular diseases in humans [34].

Although fermentative milk and milk products containing LAB have been suggested to be of special importance in human health the strains must be characterised to ensure the absence of acquired antimicrobial and antibiotic resistant properties for safe human consumption. The objective of this study is to identify the natural *L.lactis ssp.lactis* wild strains from raw milk and milk products obtained from Turkey and Iran and analyse its potential performance as a prime quality starter culture in the dairy industry. Proteolytic (casein degradation) and antimicrobial activity of *L.lactis ssp.lactis* is of special significance in determining the product quality in cheese manufacturing [27, 39, 40]. The obtained strains were examined for their proteolytic, antimicrobial activities as well as resistance to common antibiotics.

2. MATERIALS

2.1 Samples

Turkish samples used in this study included raw cow milk, white cheese and naturally processed kefir from cow milk obtained from different provinces of Turkey. Iranian samples included naturally processed cheese and yogurt from sheep milk and lor from cow milk. All samples were collected during April to June 2016 and transported to Konya, Turkey (Parmapark, Teknokent Microbiology Research Center) under refrigerated conditions in sterile 20 ml falcon tubes and were stored at 4°C until analyses. Out of 31 dairy samples collected from Iran, 21 *L.lactis ssp.lactis* isolates (cheese 13, yogurt 7, lor 1) were obtained and 77 *L.lactis ssp.lactis* isolates (kefir 2 , raw milk 57, cheese 18) were obtained from 231 dairy samples collected from Turkey and analysed within the scope of this study.

2.2 Bacterial Strains and Culture Media

Bacterial strains were isolated by the routine microbiological procedure and inoculation on a solid medium.Selective media, MRS and M17 plates were used and the cultivation of lactic acid bacteria was carried out at appropriate temperatures of 32 and 40°C for 1-3 days. On visualizing the colonies, they were selected from the highest dilution of MRS and M17 agar plates and the strains were grown in MRS broth at 25°C [22].

2.3 Identification of the Isolates

Identification of these isolates was carried out using microscopical, morphological and bio-chemical tests as suggested by Khemaria *et al*. [27]. Cell morphology of the isolates and their arrangements (diplo, chain, tetrad forms) were examined after simple staining under light microscope. All isolates were checked for catalase activity. The catalase test was carried out by the application of 3–4 drops of 3% (v/v) H2O2 to the bacterial colonies and catalase activity was confirmed by the observation of gas bubbles. The catalase-negative isolates (*L.lactis ssp.lactis*) were further subjected to molecular identification by polymerase chain reaction (PCR) according to the protocol of promega genes kit. To detect *L.lactis ssp.lactis* isolates a pair of specific universal primers: (LacF)5'GTACTTGTACCGACTGGAT-3' and (Lacre-R) 5'GGGATCATCTTTGAGTGAT-3' were used. The amplification were done according to method suggested by Buyukyoruk *et al*. [8] and Pu *et al*. [33].The PCR products (5 µl) were subjected to electrophoresis on 1% agarose gels(100V,1h).the gel was stained with ethidium bromide and observed under UV transilluminator for the presence of DNA bands [18].

2.4 Proteolitic Activity of L.lactis ssp.lactis

The isolated *L.lactis ssp.lactis* were screened for protease production using skim milk agar medium (SMA, Merck 1.15338). All isolates were streaked on to skim milk agar plates (10 µl) and the plates were incubated for 48 h at 37°C. The clear zone around the streak of bacteria was evaluated as protease producers and the diameter of inhibition zones was measured [13].

2.5 Litmus Milk Reaction of L.lactis ssp.lactis

The litmus milk reaction was tested in 5 ml of broth-type medium. Tubes containing the litmus milk were inoculated with a fresh culture of the unknown strain. The inoculum consisted of 1 drop of a 24-h broth culture. The tubes were incubated at 35°C for up to 7 days. Acid formation was read as positive when a red or white color appeared. A negative litmus milk reaction was recorded when the tube contents remained blue [15].

2.6 Total Titratable Acidity of L.lactis ssp.lactis

The total titratable acidity of *L.lactis ssp.lactis* isolates was quantitatively estimated as % lactic acid by a titrimetric method using an aliquot of 5 ml broth culture, 0.1 mol/l NaOH and phenolphthalein as indicator. The appearance of pink colouration of broth indicated the neutralisation of total acid by NaOH. The titre value of NaOH was noted and total acidity as % lactic acid (equivalent weight 90) was calculated by the following formula used Khemariya *et al*. [27].

2.7 Anti-Microbial Activity of L.lactis ssp.lactis

Anti-microbial activity of *L.lactis ssp.lactis* isolates was determined by agar well diffusion method [1]. The cell free supernatants obtained after the cultivation of *L.lactis ssp.lactis* in the sample medium and centrifugation at 1200 g for 10 minutes. *E.coli ATCC* 35218, *S.aureus* ATCC 25923, *L.sakei* ATCC15521, and *E. faecalis* ATCC 29212 were used as

target pathogens to test the antibacterial activity.The anti-microbial activity was assessed by measuring the diameter of the inhibition zone around the well.

2.8 Antibiotic Susceptibility of L.lactis ssp.lactis

The antibiotic susceptibility was measured by disc diffusion method. A total of 9 different types of antibiotics Ampicillin (AM), Erythromycin (E), Chloramphenicol (C), Gentamicin (CN), Vancomycin (VA),Clindamycin (DA),Kanamycin (K),Tetracycline (TE), Streptomycin (S) discs were used.The diameter of inhibition zones was measured. Resistance was defined as the absence of inhibition zone around the discs [27].

2.9 Detection of Streptomycin (strA and strB) and Tetracycline (tet M and tet S) Resistance Genes by PCR

In this study, *L.lactis ssp.lactis* was found to have reduced susceptibility for streptomycin and tetracycline and therefore PCR were conducted to screen the isolates with presence of resistance genes. *Str*(A)-Forward (F) 5'CTTGGTGATAACGGCAATTC3', *str*(A)-Reverse (R) 5'CCAATCGCAGATAGAAGGC3' and *str*(B)-F 5'ATCGTCAAGGGATTGAAACC3', *str*(B)-R 5'GGATCGTAGAACATATTGGC3' primers were used for amplification of streptomycin resistant genes [9]. Similarly, for tetracycline, *tet*(M)-Forward (F) 5'GGTGAACATCATAGACACGC3',*tet*(M)-Reverse (R) 5'CTTGTTC AGTTCCAATGC3' and *tet*(S)-Forward (F) 5'ATCAAGATATTAAGGAC3', *tet*(S)-Reverse (R) 5'TTCTCTATGTGGTAATC3' primers were used [42]. The PCR products were subjected to electrophoresis on 1% agarose gels (100 V, 1 hr) and the products were visualized by staining with ethidium bromide [20, 29, 43].

2.10 Statistical Analyses

IBM SPSS Statistics 22 (IBM SPSS, Turkey) program was used for statistical analyses of the data. Shapiro Wilks test was used to evaluate whether the parameters exhibited a normal distribution. Descriptive statistics as arithmetic mean, standard deviation and frequencies were calculated. For quantitative data which did not exhibit a normal distribution, Kuskal Wallis test was applied to compare data between groups and Mann Whitney U test was used to find the group causing the difference. For qualitative data, Ki-square test and Fisher's Exact test was used to compare the groups. Yates correction for continuity was used and level of significance was accepted as ($p<0.05$).

3. RESULTS & DISCUSSION

3.1 Obtaining the Properties of Bacterial Strains Collected from Different Sources

The most frequently identified species of LAB in raw milk are *Lactococcus lactis*, *Lactobacillus brevis* and *Lactobacillus fermentum. Lactococcus lactis* strains being thermoduric survive and grow rapidly in cheese during ripening as well as play an important role in aroma production and flavor development [5].

Of the 31 Iranian samples, most number of *L.lactis ssp.lactis* isolates could be obtained from cheese (13 out of 18 samples) followed by yogurt (7 out of 7) and lor (1 out of 6) samples. In case of Turkish samples (231), 18 *L.lactis ssp.lactis* isolates could be obtained out of 54 cheese samples, 57 out of 171 milk samples and 2 icolates out of 6 kefir samples. Although Iranian samples were much lesser in number, 67.7% of Iranian samples produced isolates as compared to 33.3% in Turkish samples.

Lactic acid bacteria predominate in raw milk samples universally, however, variations in microflora seem primarily due to geographical, environmental and milk compositional differences among different milk species [2]. *L.lactis ssp.lactis* is among the most predominant strains present in Lighvan, a traditional Iranian cheese varieties made from raw sheep milk owing its popularity to its typical piquant flavour [12]. The difference in *L.lactis ssp.lactis* content between Turkish and Iranian cheese may be due to the fact that one was from sheep milk (Iranian) and the other (Turkish) was obtained from cow milk. Furthermore, in some cheese varieties acidic pH resistant, hetero fermentative LAB species such as *Lactobacillus casei*, *L.paracasei* and *L.plantarum* multiply constitute the flora thereby competing with *L.lactis ssp.lactis* strains and inhibiting its growth [4, 30, 31].

L.lactis ssp.lactis strains were not able to be isolated in some milk samples probably because of factors such as antibiotic contaminations and bacterial inhibitions due to sterilizing agents used in product tankers and containers, suppressive activities of free fatty acids, antimicrobial proteins and lacto peroxidases present in raw milk [37, 41]. Under microscope, morphology of the isolated strains in this study represented cocci assembled in pairs or short chains of various length possessing 2, 4 or 12 cells.

3.2 Skim Milk Agar Test

Skim milk agar test, conducted to determine the proteolytic activity of the isolated *L.lactis ssp.lactis* in the samples, were evaluated by measuring the opaque inhibition zones around the bacterial streak. Of all the samples studied, one of the Iranian cheese isolate and five of the Turkish milk isolates did not exhibit any proteolytic activity. Difference between the proteolytic activity measured as opaque zone diameter of cheese samples from Turkey and Iran were found

to be statistically insignificant (p>0.05). The opaque zone diameter measured in the cheese samples of Turkey were significantly higher compared to the milk samples (p:0.017; p<0.05). (Figure 1). Higher proteolytic activity of cheese compared to milk samples in general can be explained since it has been suggested that strains isolated from niches with adverse and competitive conditions as high microbial competition (raw-milk cheeses) or n scarce availability of nutrients (vegetable surfaces) demonstrate a more efficient bacterial metabolism [23].

3.3 Total Titratable Acidity of L.lactis ssp.lactis

Lactic acid production in milk results in lowering of pH and initiation of proteolytic reaction by the bacteria rendering it useful as a starter culture in the dairy industry. Acidic properties in raw and fermented milk products are considered among the quality parameters. As per the Turkish Food Codex, appropriate acid content expressed as percentage for milk products is between 0.135 – 0.2%. In this study, the lactic acid parameters were between 0.16± 0.18% and in accordance to the Turkish Food Codex. Lactic acid content found in Iranian yoghurt 0.13± 0.04% (acceptable range 0.6 – 1.5%) were also found in accordance with the Turkish Food Codex. The maximum acceptable titration acid content of white cheese is 3% as per the Codex; in this study lactic acid content of Turkish white cheese samples were in the range of 0.1± 0.02% and Iranian white cheese samples were 0.39± 0.87%.

In this study lactic acid production by Iranian cheese samples were significantly higher (0.39±0.87%) as compared to the samples in Turkey (0.1±0.02%). (p:0.032; p<0.05). (Figure 1). This difference may have resulted due to the fact that origin of Iranian cheese was sheep milk which is suggested to have higher coagulation properties as compared to cow milk since sheep milk contains more casein and colloidal calcium contributing to the property [38].

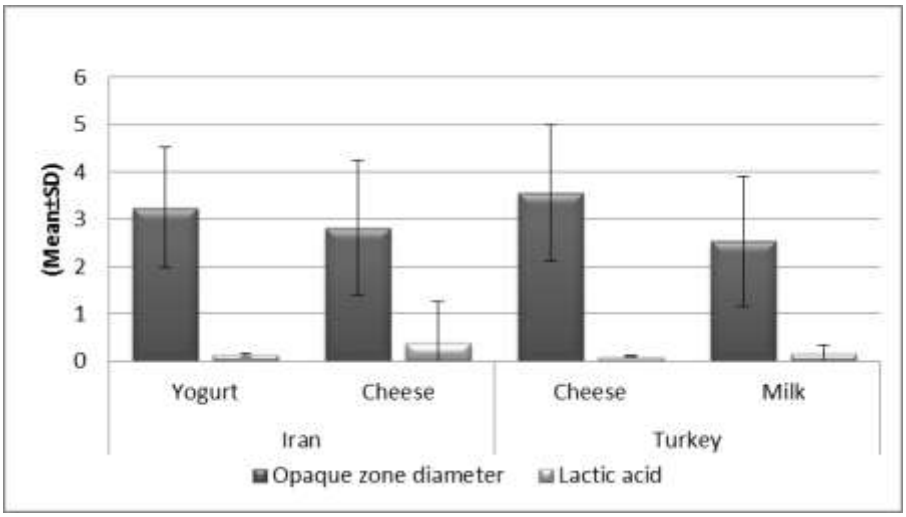

Figure 1: Proteolytic Activity of the Samples (Opaque Zone Diameter) and Lactic Acid Production

3.4 Litmus Milk Reduction Test

Litmus milk test was conducted in order to evaluate the metabolic activity *of L.lactis ssp.lactis* strains obtained from the samples.Table 1 shows the litmus milk reduction test results (indicated by presence or absence of white or pinkish white solid band) for the various isolates from Turkey and Iran. Based on these characteristics, the isolates were phenotypically identified as *L.lactis ssp.lactis* [27]. The difference between the samples, when compared on individual country basis or between countries, were found to be statistically insignificant. (p>0.05). However, phenotypic characteristics may be variable within certain genotypically similar strains of *L.lactis ssp.lactis* species due to differences in enzymatic expression [16]. Therefore, characterization of potential starter strains require phenotypic, biochemical and genotypic evaluation of bacterial strains [25].

Table 1: Evaluation of Litmus Milk Reduction Test Results for all Samples

Country	Product	Band Formation	
		Present	Absent
		n (%)	n (%)
Iran	Yoghurt	4 (%57.1)	3 (%42.9)
	Cheese	9 (%69.2)	4 (%30.8)
	[1]p	0.651	
Turkey	Cheese	6 (%33.3)	12 (%66.7)
	Milk	34 (%59.6)	23 (%40.4)
	[2]p	0.093	

[1]Fisher's Exact Test [2]Continuity (Yates)

3.5 Anti-Microbial Activity of L.lactis ssp.lactis

In this study, anti-microbial activity of *L.lactis ssp.lactis* was assessed for four common pathogenic bacterial strains (*E. coli, E. fecalis, S. aureus, L. sakei*) present in the human gastro-intestinal tract. *L.lactis ssp.lactis* is suggested to have significant role in the inhibition of pathogenic bacterial growth during cheese manufacture from non-pasteurised raw milk. Means of the anti-microbial activity of all the isolates (assessed by measuring the diameter of the inhibition zone for each bacterial specie) obtained from Iran and Turkey has been summarized in Table 2.

Among Iranian samples, antimicrobial activity of yoghurt against *E.Coli* was significantly higher as compared to S.aureus (p=0.035) and *L.sakei* (p=0.003). Antimicrobial activity of Iranian cheese was highest and statistically significant for *E.faecalis* as compared to *S.aureus* and *L.sakei* species (p<0.05. In general, among Turkish samples, both milk and cheese exhibited highest anti-microbial activity against *E. faecalis.* Activity against *S. aureus* in cheese was also found to be significantly high as compared to *E. coli* and *L. sakei* (p<0.05; Kruskal Wallis test) (Table 2). Since permeability barrier of gram negative bacterial cell envelopes are lower as compared to gram positve strains, microbial activity against *E. coli* (gram negative) was found to be less effective as compared to *S. aureus* (gram positive) as was expected [6, 7, 26]. When compared on product basis, Iranian cheese showed significantly higher activity for *E. faecalis* and *E. coli* as compared to the Turkish counterpart (p:0.022; p<0.05) (p:0.009; p<0.05; Mann Whitney U test). Marked antimicrobial activity of *L.lactis ssp.lactis* from yoghurt isolates against *E.coli* and *S. Aureus* have also been demonstrated by other studies [3, 14].

Table 2: Evaluation of Anti-Microbial Activity of all Samples

	Iran		Turkey	
	Yoghurt	Cheese	Milk	Cheese
	Mean±SD (median)		Mean±SD (median)	
E. faecalis	2.66±0.96 (2.2)	3.44±0.9 (3.5)	2.97±1.11 (2.9)	2.59±1.2 (2.3)
E. coli	3.42±0.62 (3.6)	2.46±1.73 (3.3)	0.93±1.61 (0)	0.71±1.67 (0)
S. aureus	2.07±1.06 (1.4)	2.38±0.75 (2.1)	2.58±1.03 (2.5)	1.97±0.73 (2)
L. sakei	1.73±0.87 (1.9)	1.62±0.59 (1.7)	1.21±1.23 (1.4)	1.83±1.76 (1.5)
p	0.019*	0.001*	0.001*	0.001*

*Kruskal Wallis Test *p<0.05*

3.6 Antibiotic Susceptibility of L.lactis ssp.lactis

Drug resistance presents an ever-increasing threat to public health and one of the most troubling event is the transfer of antibiotic resistant genes into pathogenic bacterial species causing infections that are effectively untreatable. In this respect determination of antibiotic resistance of *L.lactis ssp.lactis* strains is of significant importance for its potential use as a starter culture in the dairy industry.

Among Turkish cheese samples, maximum resistance was demonstrated for streptomycin (55.6%) followed by erythromycin (22.2%) and tetracyclin (11.1%). Milk samples exhibited maximum resistance to streptomycin (33.3%) followed by tetracycline (22.8%) and erythromycin (15.8%) (Table 3).

Table 3: Evaluation of Antibiotic Susceptibility/Resistance in Turkish Samples

Products		Cheese		Milk		
		S	R	S	MS	R
		n (%)	n(%)	n(%)	n(%)	n(%)
Turkey	VA	18 (%100)	0 (%0)	57 (%100)	0 (%0)	0 (%0)
	S	8 (%44.4)	10 (%55.6)	38 (%66.7)	0 (%0)	19 (%33.3)
	TE	16 (%88.9)	2 (%11.1)	42 (%73.7)	2 (%3.5)	13 (%22.8)
	K	18 (%100)	0 (%0)	57 (%100)	0 (%0)	0 (%0)
	CN	18 (%100)	0 (%0)	57 (%100)	0 (%0)	0 (%0)
	E	14 (%77.8)	4 (%22.2)	45 (%78.9)	3 (%5.3)	9 (%15.8)
	DA	18 (%100)	0 (%0)	57 (%100)	0 (%0)	0 (%0)
	C	18 (%100)	0 (%0)	56 (%100)	0 (%0)	0 (%0)
	AM	18 (%100)	0 (%0)	45 (%78.9)	7 (%12.3)	5 (%8.8)
	p	0.001*		0.001*		

Ki Kare Test **p<0.05* (S:Susceptibility; MS:Medium Susceptible; R:Resistance)

Similar to Turkish cheese samples, Iranian cheese also exhibited maximum resistance to streptomycin (23.1%) followed by tetracycline and Kanamycin (15.4%). Yogurt samples demonstrated maximum resistance to tetracycline followed by streptomycin.

Turkish and Iranian samples exhibited notable resistance to streptomycin and tetracycline. Although in Turkish samples this resistance was found to be statistically significant (p:0.048, p<0.05) as compared to other antibiotics, for Iranian samples they were not statistically significant (p>0.05). (Table 4).

Table 4: Evaluation of Antibiotic Susceptibility/Resistance in Iran Samples

Products		Yogurt			Cheese		
		S	MS	R	S	MS	R
		n(%)	n (%)	n(%)	n(%)	n(%)	n(%)
Iran	VA	7(%100)	0 (%0)	0 (%0)	13 (%100)	0 (%0)	0 (%0)
	S	6(%85.7)	0 (%0)	1 (%14.3)	10 (%76.9)	0 (%0)	3 (%23.1)
	TE	5(%71.4)	0 (%0)	2 (%28.6)	11 (%84.6)	0 (%0)	2 (%15.4)
	K	7(%100)	0 (%0)	0 (%0)	13 (%100)	0 (%0)	2 (%15.4)
	CN	7(%100)	0 (%0)	0 (%0)	13 (%100)	0 (%0)	0 (%0)
	E	4(%57.1)	3 (%42.9)	0 (%0)	12 (%92.3)	1 (%7.7)	0 (%0)
	DA	7(%100)	0 (%0)	0 (%0)	13 (%100)	0 (%0)	0 (%0)
	C	7(%100)	0 (%0)	0 (%0)	13 (%100)	0 (%0)	0 (%0)
	AM	6(%85.7)	1 (%14.3)	0 (%0)	12 (%92.3)	1 (%7.7)	0 (%0)
	p	0.010*			0.055		

Ki Kare Test **p<0.05* (S:Susceptibility; MS:Medium Susceptible; R:Resistance)

3.7 Detection of Tetracycline (tet M and tet S) and Streptomycin (strA and strB) Resistance Genes by PCR

The genetic basis of the observed phenotypic resistance to tetracycline and streptomycin resistance as indicated by the disc diffusion method was further confirmed by polymerase chain reaction (PCR). The specific primers of Tet (S), Tet (M), Str(A) and Str (B) were used to identify the presence of tetracycline and streptomycin resistance genes. The antibiotic resistance for the bacterial isolates under study are shown on Agarose Gel Electrophoresis after PCR amplification in Figure 2 none of the *L.lactis ssp.lactis* Iranian isolates exhibited antibiotic resistance. Among the Turkish *L.lactis ssp.lactis* isolates, Tet (S) resistance gene was confirmed in 6 milk and 1 cheese isolates, Str (A) resistance gene was confirmed in 6 milk and 1 cheese isolates and Str (b) resistance gene was confirmed by PCR in only 1 milk isolate (Figure 2).

In a study with *L.lactis ssp.lactis* strain isolated from raw milk and soft cheese, the strain demonstrated resistance to streptomycin, chloramphenicol and tetracycline [35]. In order to study Gómez *et al*. [21] all strains of *L.lactis ssp.lactis* were sensitive to tetracycline, ampicillin and erythromycin and resistant to vancomycin. In this study however, through PCR amplification, Tet (S) resistance gene was confirmed in 6 milk and 1 cheese isolates, Str (A) resistance gene was confirmed in 6 milk and 1 cheese isolates and Str (b) resistance gene was confirmed by PCR in only one milk isolate (Figure 2). Similar to the findings of this study, Gad *et al*. [18] in his study with 244 LAB strains isolated from dairy and pharmaceutical products, 29.6% of *L.lactis ssp.lactis* isolates showed resistance to tetracyclin. Partial resistance to tetracyclin (12%) and streptomycin (5.4%) was also demonstrated by Devirgilis *et al*. [11] and Florez *et al*. [17] , working with several isolates from natural environments. Resistance to vancomycin, erythromycin and streptomycin) were detected in some of the isolates.

These results overlap with the results reported by other research groups, indicating that most of the antibiotic-resistant LAB strains are resistant to tetracycline and erythromycin. The reason for this may be due to the huge amount of tetracyclines that have been used for years in agriculture livestock farming as "growth promoters" [43]. Tetracyclines continue to be important as therapeutic antibiotics, and are still employed in stockbreeding and aquaculture in many countries [17]. Antibiotic resistance genes (ARGs) are found not only in the clinical but also the natural environment, which can eventually produce antibiotic resistant bacteria (ARB). Antibiotics and ARB are released to the environment from hospitals, livestock facilities, and sewage treatment plants (STP) [36]. Therefore, it is very important to verify that the nutritional LAB strains consumed on a daily basis lack acquired antimicrobial resistance properties prior to considering them safe for human and animal consumption [3]. In this respect, however, it is important to realize that acquired antibiotic resistances can be transferred not only by conjugation but also by other mechanisms, such as transformation or transduction, that are even more difficult to study under controlled laboratory conditions [29].

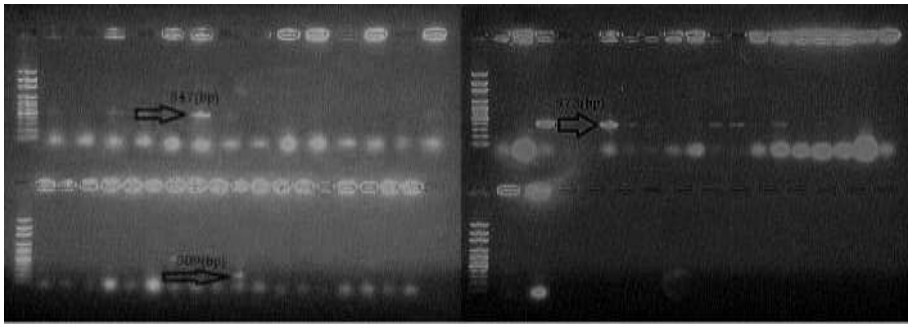

Figure 2: PCR Amplification of Tet (M)/Tet (S) and Str (A)/Str (B) Resistance Genes in the Strains of *L.lactis ssp.lactis*

3.8 Detection of Multi Resistance Genes in L.lactis ssp.lactis strains

In one of the bacteria isolates which was extracted from cheese, 2 resistance genes to antibiotics were stabilized by PCR experiment. These isolates are multi resistance and have resistance gene to antibiotics Streptomycin A (StrA) and Streptomycin B (StrB). Also 2 cases of the isolated bacteria of raw milk are also multi resistance and they have 2 resistance genes to antibiotics Tetracycline (Tets) and Streptomycin (StrA).

3.9 Selective Isolates for Potential Starter Culture

In accomplished research on 98 *L.lactis ssp.lactis* bacteria isolates, 1 isolates of Kefir and 1 isolate of Turkish cheese have antimicrobial activity against each 4 pathogen bacteria, positive metabolic activity and the ability for making curd, positive proteolytic activity, ability for lactic acid production (less than %3 and in 0.135-0.2% area) and also lack of resistance genes to Tetracycline and Streptomycin. 2 noted isolates toward other isolates are suitable choices for using in the industry of milk and cheese making.

4. LIMITATION OF THE STUDY

The major limitation of the study was the availability of fewer number of Iranian samples as compared to the Turkish ones. Although non-parametric tests were conducted to evaluate the data, further studies with large number of Iranian milk products are proposed for further comparisons and increased reliability of the study.

5. CONCLUSION

The results showed that *L.lactis ssp.lactis* isolated from cheese samples obtained from sheep milk demonstrated better antimicrobial activity and lactic acid production. Therefore emphasis must be given on the importance of selection of the milk source for obtaining good quality starter cultures. Determination of high quality starter cultures, by obtaining and evaluating the properties of bacterial strains collected from multiple regions, different seasons and sources and comparison of data may provide important advantages for the food industry.

Further studies may be conducted to learn the effect of ecological parameters such as plant/animal origin, seasonal modifications during isolation, environmental stress and similar factors on the technolical properties of the bacterial strain. Since *L.lactis ssp.lactis* strain possesses GRAS status, further investigation regarding selection of the most appropriate strain for food sector and knowledge about the virulent genes and other molecular analysis regarding its microbiota is of critical importance for public health.

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