Kafkas Univ Vet Fak Derg
24 (3): 443-450, 2018
DOI: 10.9775/kvfd.2017.19196

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

Investigation of Some Biochemical Properties, Antimicrobial Activity and Antibiotic Resistances of Kefir Supernatants and *Lactococcus lactis* ssp. *lactis* Strains Isolated from Raw Cow Milk and Cheese Samples

Shila VAHABZADEH [1] Haydar ÖZPINAR [1]

[1] Istanbul Aydin Univeristy, Department of Food Safety and Nutrition, TR-34295 Istanbul - TURKEY

Article Code: KVFD-2017-19196 Received: 10.12.2017 Accepted: 12.03.2018 Published Online: 13.03.2018

How to Cite This Article

Vahabzadeh S, Özpınar H: Investigation of some biochemical properties, antimicrobial activity and antibiotic resistances of kefir supernatants and *Lactococcus lactis* ssp. *lactis* strains isolated from raw cow milk and cheese samples . *Kafkas Univ Vet Fak Derg,* 24 (3): 443-450, 2018. DOI: 10.9775/kvfd.2017.19196

Abstract

In this study some biochemical characteristics, antibiotic resistance profiles and antibacterial activity of kefir microfloras (kefir supernatants) and *Lactococcus lactis* ssp. *lactis (L. lactis* ssp. *lactis)* bacteria isolated from cheese made from sheep milk and raw cow milk were investigated. In this study, 30 kefir supernatants and 60 isolates of *L. lactis* ssp. *lactis* were analyzed. 30 isolates obtained from raw cow milk and 30 isolates obtained from raw sheep cheeses. In kefir supernatants the highest antimicrobial activity was observed against *B. cereus*, followed by *E. coli, S. aureus* whereas no antimicrobial activity was observed against the *E. faecalis*. Kefir supernatants exhibited highest resistance to chloramphenicol and lowest resistance to erythromycin. The highest antimicrobial activity in *L. lactis* ssp. *lactis* isolates obtained from raw sheep cheese and raw cow milk samples were found against *E. faecalis* followed by *S. aureus, L. sakei* and *E. coli* respectively. The isolates obtained from raw sheep cheese samples 43.3% were resistant to streptomycin, 13.3% tetracycline and 22.2% erythromycin. The isolates obtained from raw cow milk samples 40% were resistant to streptomycin, 40% tetracycline, 16.7% ampicillin and 23.3% erythromycin. The results showed that *L. lactis ssp. lactis* bacteria isolated from cheese samples derived from raw sheep milk demonstrated better antimicrobial activity and lactic acid production. Among all isolates obtained from raw cow milks and raw sheep cheeses Tet(S) resistance gene was confirmed in 7 (11.7%), Str(A) in 6 (10%) and Str(B) in 1 (1.7%) isolates by PCR method.

Keywords: *L. lactis ssp. lactis, Kefir, Dairy products, Antimicrobial activity, Antibiotic resistance*

Çiğ Süt, Peynir Örneklerinden İzole Edilen *Lactococcus. lactis* ssp. *lactis* Suşlarının ve Kefir Süpernatanlarının Bazı Biyokimyasal Özellikleri, Antimikrobiyel Etkinliği ve Antibiyotiklere Karşı Dirençlığının Araştırılması

Öz

Bu çalışmada çiğ koyun sütünden yapılan peynir örneklerinden ve inek sütünden izole edilen *Lactococcus lactis* ssp. *lactis (L. lactis* ssp. *lactis)* izolatlarının ve kefir süpernatanlarının (kefir mikroflorasi) bazı biyokimyasal özellikleri, antibiyotiklere karşı dirençliliği ve antibakteriyal etkileri incelenmiştir. Araştırmada 30 kefir süpernatantı ve 60 *L. lactis* ssp. *lactis* izolatı analiz edilmiştir. 30 izolat çiğ inek sütlerinden ve 30 izolat koyun peyniri örneklerinden elde edilmiştir. Kefir süpernatanlarının *E. faecalis*'e karşı antimikrobiyal etki göstermezken, *B. cereus*'a karşı en etkili olduğu gözlenmiştir. Ayrıca şiddete sırasına göre *E. coli* ve *S. aureus*'a etkili olduğu saptanmıştır. Kefir süpernatantlarinda antibiyotiklere karşı en yüksek direnç kloramfenikol ve en düşük direnç ise eritromisin'de gözlenmiştir. Çiğ koyun peyniri örneklerinden ve çiğ inek sütlerinden izole edilen *L. lactis* ssp. *lactis* izolatlarının en yüksek antimikrobiyal etkisi *E. faecalis*'e karşı gözlenmiştir. Ayrıca *S. auerus* ve *L. sakei*'ye karşı antimikrobiyal aktiviteleri *E. coli* göre daha yüksek olduğu görülmüştür. Çiğ koyun peyniri örneklerinden izole edilen *L. lactis* ssp. *lactis* izolatların antibiyotiklere karşı %43.3 streptomysine, %13.3 tetrasikline ve %22.2 eritromisine dirençli bulunmuştur. Çiğ inek süt örneklerinden elde edilen *L. lactis* ssp. *lactis* izolatların'da ise %40 streptomysine, %40 tetrasikline, %16.7 ampisiline ve %23.3 eritromisine karşı dirençli bulunmuştur. Sonuç olarak, koyun peyniri örneklerinden izole edilen *L. lactis* ssp. *lactis* izolatlarının daha yüksek antimikrobiyal aktivite ve laktik asit üretimine sahip olduğu saptanmıştır. Çiğ inek sütü ve çiğ koyun peynirlerden elde edilen tüm izolatlar arasında, 7 adetinde Tet(S) (%11.7) , 6 adetinde Str(A) (%10) ve 1 adetinde Str(B) (%1.7) direnç geni PCR metoduyla ile saptanmıştır.

Anahtar sözcükler: *L. lactis ssp. lactis, Kefir, Süt ürünleri, Antimikrobiyal aktivite, Antibiyotik direnci*

İletişim (Correspondence)
+90 539 7941609 (S. Vahapzadeh), +90 545 9173416 (H. Özpınar)
shilavahabzadeh@gmail.com, haydarozpinar@aydin.edu.tr

INTRODUCTION

Kefir is a cultured, fermented milk beverage made with kefir grains or "starter culture" which is a combination of yeasts, milk proteins, and bacteria also known as the "kefiran" complex basically a glucogalactan produced by *Lactobacillus kefirofaciens*. Kefir has a tart, creamy flavor and apart from having a high nutritional value, it is also known to have a probiotic effect [1]. Lactic acid bacteria (LAB) species including *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus* as well as acetic acid bacteria from the genus *Acetobacter* have been identified in kefir grains [2]. Kefir is usually produced from cow milk although goat and sheep milk can also be used for its production [3]. The kefir grains collected from different geographical areas exhibit different microbial flora [4]. Among the major health benefits of kefir; improvement of lactose intolerance by altering the microbial flora of the intestinal system [5], antibacterial properties against fungi, gram positive and negative bacteria due to the presence of hydrogen peroxide, lactic acid, carbon dioxide, alcohol, diacetyl, bacteriocins and acetaldehyde as well as its anti-carcinogenic properties are worth mentioning [6]. Since the pathogens have been reported to have developed resistance to antibiotics in recent years, utilizing the antimicrobial factors as in the case of kefir, for coping against pathogens in dairy products is of special significance [7]. Milk peptides released due to the activity of protease enzymes produced by lactic acid bacteria during kefir fermentation causes easy digestion of milk proteins and enhances the organoleptic properties in dairy products [8]. Selection of good starter cultures for fermented dairy products is of particular importance and use of kefir as a starter culture in fermented milk products, especially in cheese production, is being widely practiced in recent years and several studies indicate that the degree of proteolysis and lactic acid concentration are higher in cheese produced by kefir as compared to other starter cultures [9]. Another member of the LAB family which is commonly termed generally recognized as safe (GRAS) also playing an important role as starter cultures in industrial dairy fermentation is *Lactococcus lactis* ssp. *lactis (L. lactis* ssp. *lactis)* and can be found in raw cow milk [10] and sheep milk [11]. Owing to its pH lowering effect and antimicrobial metabolite production potential, it is used in the dairy industry as an additive; enhancing the organoleptic attributes, shelf life and product quality. In addition, *L. lactis* ssp. *lactis* also plays a major role in ripening of cheese matrix due to its ability to produce intracellular peptidase enzymes [12]. Selected *L. lactis* ssp. *lactis* strains are suggested to favorably influence the intestinal flora of human and animal hosts, lead to competitive exclusion of gastrointestinal pathogens, stimulate immune responses and exhibit anti-carcinogenic properties [13]. Dairy products containing *L. lactis* ssp. *lactis* strains have been found to improve digestion and absorption of lactose sugar in lactose intolerant individuals, reduce heart rate and blood pressure in hypertensive rats and prove beneficial in the prevention and treatment of cardiovascular diseases in humans [14]. Since the microbial flora and profile of starter culture directly influence the organoleptic and physiochemical characteristics of dairy products as cheese made from raw milk, study of this bacterium's metabolic activity in vitro will elucidate the characteristics and potential performance as a prime quality starter culture in the dairy industry [15]. Isolation of prime quality starter cultures with high metabolic activity from natural resources and its industrialization has brought about a modern revolution in the dairy products. Kefir is the most popular fermented milk product which is consumed around the world and *L. lactis* ssp. *lactis* is one of the most bacteria that used in dairy industry. The aim of this research is to identify the some properties of *L. lactis* ssp. *lactis* isolated from raw milk, cheese samples and kefir supernatants by analyzing its metabolic activity, sensitivity to antibiotics, lactic acid production as well as the antimicrobial activity.

MATERIAL and METHODS

Materials

The 60 isolates of *L. lactis* ssp. *lactis* from raw cow milk (30) and cheese made from raw sheep milk (30) were collected in *our previous study which performed* at Farmapark Biotechnology Research and Development Ltd. located in Konya, Turkey in June and July 2016. A total of 30 kefir samples were collected in May 2016 from various cities in Turkey (Bursa, Canakkale, Istanbul, Konya, Kocaeli, Tokat, Sakarya, Tekirdag and Amasya). In this study all *control organisms* including *Enterococcus faecalis* ATCC 29212 *(E. faecalis* ATCC 29212*), Escherichia coli* ATCC 35218 *(E. coli* ATCC 35218*), Staphylococcus aureus* ATCC 25923 *(S. aureus* ATCC 25923*) and Lactobacillus sakei* ssp. *sakei* ATCC15521 *(L. sakei* ssp. *sakei* ATCC15521). *Bacillus cereus (B. cereus)*, *Listeria monocytogenes (L. monocytogenes*) were obtained from Farmapark Biotechnology Research and Development Ltd.

Methods

Samples: Kefir samples were collected into 50 mL sterile falcon tubes and transported to the laboratory *under cold conditions* and stored at 4°C until analysis. All isolates of *L. lactis* ssp.*lactis* were kept at MRS broth medium containing 20% glycerol and preserved at -20°C [16]. The analyses were carried out in Farmapark Biotechnology Research and Development Ltd.

Preparation of Kefir Samples for Analysis: For analyses, 30 samples of kefir were centrifuged for 20 min at 6000 rpm then each *supernatant* was filtered through sterile filter paper. The supernatants was stored in eppendorf tubes at 4°C and used for analysis of metabolic and antimicrobial activity, lactic acid production and disc diffusion test.

Preparation of L. lactis ssp. lactis Isolates: A 10 µL (loop

full) of each broth culture was streak on MRS agar medium and incubated 24-48 h at 30°C. Once single colonies were observed, they were inoculated in MRS broth and incubated at 30°C for 24 h. Gram stain and catalase test were utilized for verify the purity of bacterial cultures. *Lactococcus. lactis* ssp. *lactis* was found to be catalase negative and were observed in cocci forms under microscope [17]. Isolates were kept at MRS broth medium containing 20% glycerol and preserved at -20°C for molecular identification.

Molecular Identification of L. lactis ssp. lactis Isolates: *DNA extraction was done according* to the protocol of promega wizard® genomic DNA purification kit. PCR test was carried out using a specific pair of primer (Lac-F:5' GTACTTGTACCGACTGGAT-3', Lacre-R:5'GGGATCATCTTTA GTGAT-3') for molecular identification of *L. lactis* ssp. *lactis* isolates. The amplification cycle included an initial denaturation of 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 58°C for 40 s and extension at 72°C for 1 min [18]. The 60 isolates of *L. lactis* ssp. *lactis* from raw cow milks (30) and cheeses made from raw sheep milk (30) were identified as *L. lactis* ssp. *lactis* by PCR.

Metabolic Activity: For evaluation of metabolic activity with pH changes at different times, 0.2 mL (200 µL) of each kefir supernatant was added to 10 mL skimmed milk (12% concentration) and incubated at 30°C. The pH of the solution were recorded with pH digital thermometer (WTV735I, Germany) after 6, 12, 18, 24 and 30 h. Litmus milk reaction test was used for observing the metabolic activity of *L. lactis* ssp. *lactis* isolates obtained from milk and cheese samples. Five mL of litmus milk medium were added to each test tube and 0.1 mL of each culture bacterium was taken from MRS broth with pipette and was cultured into the litmus milk medium. The tubes were incubated at 30°C 24 to 48 h. A pinkish color or a pinkish color white curdy appearance, due to acidic medium produced, indicated positive results. Stabilized blue and no change shows the test negative [19].

Lactic Acid Production: One mL of each broth culture at a turbidity of 0.5 McFarland standards and kefir supernatants was inoculated to 9 mL sterile skimmed milk medium and incubated at 30°C for 6 h. After 6 h incubation period, 0.5 mL phenolphthalein was added to each tube as indicator and titrated with 0.1 N NaOH until a pink colour of indicator persists for at least 30 s. The amount of consumed NaOH was recorded for each sample. In the end, the amount of lactic acid produced by each *L. lactis* ssp. *lactis* isolates and kefir supernatants were measured by the formula suggested by Khemariya et al.[17].

Total acidity (%) = (titre value × normality of alkali × volume made up × equivalent weight of acid × 100)/(aliquot taken × weight/volume of sample × 1000)

Antimicrobial Activity: In this study antimicrobial activity of *L. lactis* ssp. *lactis* isolates obtained from milk, cheese, and kefir supernatants were investigated by using agar well diffusion method. Various type of gram positive and negative bacterial species were selected for observing antimicrobial activity of *L. lactis* ssp. *lactis* isolates including *E. faecalis, E. coli, S. aureus* and *L. sakei* ssp. *sakei*. *L. sakei* ssp. *sakei* which is a bacteriocin sensitive indicator (non-bacteriocin producer), was used in this study for investigating the antimicrobial activity of *L. lactis* ssp. *lactis* isolates obtained from milk and cheese samples.

Staphylococcus aureus, E. faecalis, *E. coli strains* commonly used as a control strain for routine antimicrobial susceptibility tests. *Bacillus cereus*, *L. monocytogenes, E. coli, S. aureus* and *E. faecalis* were used for evaluating the antimicrobial activity of kefir supernatants. *Bacillus cereus* and *L. monocytogenes* also listed among the pathogens known to cause serious health problem in hosts were taken into consideration while working with kefir. The test microorganisms were grown in nutrient broth diluted in Muellller-Hinton Broth (MHB) to equal the turbidity of the 0.5 McFarland standard ($1.5x10^8$ cfu/mL) then spreading on Mueller Hinton Agar (MHA) by using steril cotton swab. Wells, 8 mm in diameter, were punched in the agar plates. The *L. lactis* ssp. *lactis* isolates were cultured in MRS broth centrifuged for 10 min per 1200 *g* then 200 µL of each supernatant was inoculated into the wells. All petri dishes were incubated for 24-30 h at 30°C. The diameter of clear zones around every well was measured and recorded [20].

Determination of Antibiotic Resistance: Disc diffusion test was done according to the instruction of national committee for clinically laboratory standards (NCCLS). Kanamycin, tetracycline, streptomycin, vancomycin, clindamycin, erythromycin, ampicillin, gentamicin, chloramphenicol antibiotics were selected for the test [21]. *Lactococcus lactis* ssp. *lactis* isolates were grown in MRS broth were diluted in MHB to equal the turbidity of the 0.5 McFarland standard then inoculated on MHA by using steril cotton swab. All plates left to dry at room temperature for 15 min. Antibiotic discs were placed on the inoculated plates by using sterile forceps and incubated for 24 h at 30°C. Each kefir supernatant was swabbed on MHA. Diameter of clear zone around antibiotic discs were measured. Lack of inhibition zones around disc of each antibiotic exhibited resistance to it [17].

Detecting of Tetracycline and Streptomycin Resistance Genes: Two specific pairs of primers namely Tet(M), Tet(S) and Str(A), Str(B) were used to identify tetracycline and streptomycin resistance genes. The PCR program for streptomycin consisted of denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min. A final extension step at 72°C for 10 min ended the PCR protocol. The sequence of primers includes: Str(A)(F)5'CTTGGTGATAAC GGCAATTC3', Str(A)(R)5'CCAATCGCAGATAGAGGC3', Str(B)(F) 5'ATCGTCAAGGGATTGAAACC3', Str(B)(R)5'GGATCGTAGAAC

ATATTGGC3'. PCR program for tetracycline consisted of denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, annealing at 55°C for 1 min for the following primers. Tet(M) with a sequence Tet(M)(F)5' GGTGAACATCATAGACACGC3', Tet(M)(R)5›CTTGTTCAGTTC CAATGC3' and Tet(S)(F)5'ATCAAGATATTAAGGAC3', Tet(S) (R)5›TTCTCTATGTGGTAATC3' and extension at 72°C for 2 min. A final extension step at 72°C for 7 min ended the PCR protocol. 5 µL of each DNA were added to 1% agarose gel and electrophoresis was applied for 1 h at 100 volt (100V,1 h). The gel was observed under UV light after being colored with ethidium bromide. This PCR resulted in a DNA fragment of 548 and 509 base pairs for Str(A) and Str(B) and 401, 573 base pairs for Tet(M) and Tet(S) resistance genes [22,23]. Primers were obtained from BM Labosis LTD.located in Istanbul, Turkey.

Statistical Analysis

In this study, IBM SPSS Statistics 22.0 version (IBM SPSS, Turkey) was used to analyze the data statistically. In order to compare the data which had normal distribution, shapiro wilks and student T-test were conducted. One-way anova test, chi-square test and fisher's exact test were performed to compare the quantitative date between the groups.

RESULTS

Metabolic Activity: Metabolic activity of 30 kefir super-natants were evaluated by pH changes during fermentation at various time intervals. The minimum pH was seen in 30 h. The results demonstrated that pH decreased with time *(Table 1)*. Metabolic activity was evaluated using litmus milk reaction test in *L. lactis* ssp. *lactis* isolates. Among 30 isolates obtained from cheese samples, curdy appearance was created in 10 isolates (33%) and in isolates obtained from raw milk, curd was created in 26 isolates (86.7%).

Lactic Acid Production: Among 30 kefir supernatants, the amount lactic acid produced was in the range of 0.23-0.39% and was observed with a mean value of 0.31%. In the 30 isolates of *L. lactis* ssp. *lactis* obtained from cheese samples, the amount of lactic acid production was observed in the range of 0.03% to 0.90%, mean value recorded as 0.14±0.16% and In the 30 isolates of *L. lactis* ssp. *lactis* obtained from milk samples, the amount of lactic acid production was observed in the range of 0.06% to 0.36% mean value recorded as 0.12±0.07%.

***Table 1**. Evaluation of metabolic activity in kefir supernatants with pH value during fermentation*

Time	Min-Max	Mean±SD
6th h	6.05-6.43	6.24±0.12
12th h	5.46-6.5	5.96±0.23
18th h	5.07-6.33	5.77±0.27
24th h	4.77-6.17	5.44±0.28
30th h	4.01-5.88	4.93±0.35
P	0.000*	

** P<0.05*

***Table 2.** Evaluation of antibiotic resistance in kefir supernatants by disc diffusion method (The mean value of the clear zones around antibiotic discs in mm)*

Antibiotic	Min-Max	Mean±SD
VA[1]	0.23-4.91	2.69±1.28
ST[2]	3.02-7.96	5.16±1.30
TE[3]	4.66-9.67	7.18±1.49
KA[4]	0.19-4.83	2.39±1.28
CN[5]	4.54-9.39	6.60±1.25
ER[6]	5.09-9.71	7.24±1.21
DA[7]	2.39-6.82	4.48±1.28
CH[8]	0.03-3.96	1.90±1.05
AM[9]	0.1-6.99	3.94±1.87
P	0.000*	

** P<0.05; VA[1]: Vancomycin, ST[2]: Streptomycin, TE[3]: Tetracycline, KA[4]: Kanamycin, CN[5]: Gentamicin, ER[6]: Erythromycin, DA[7]: Clindamycin, CH[8]: Chloramphenicol, AM[9]: Ampicillin*

Antibiotic Resistance: The creation of clear zones around the antibiotic discs represented the sensitivity of kefir supernatants to the antibiotics. *Table 2* indicates the values of sensitivity spectrum of kefir supernatants to the antibiotics. The highest sensitivity values were observed against the erythromycin antibiotics. The sensitivity of chloramphenicol antibiotic was the lowest in contrast to the other antibiotics being significantly different from the others. Statistical analyses indicated resistance of some of the *L. lactis* ssp. *lactis* isolates against streptomycin, tetracycline, ampicillin and erythromycin *(Table 3)*.

Antimicrobial Activity: On examining the antimicrobial activity of kefir supernatants, the highest antimicrobial activity was demonstrated against the *B. cereus* (33.3%) while no effect was seen on *E. faecalis*. Antimicrobial activity of kefir supernatants against the *E. coli* (13.3%) in contrast to the *S. aureus* (3.3%) was significantly higher. 86.7% of kefir supernatants lacked antimicrobial activity against the *L. monocytogenes* and overall 13.3% of supernatants exhibited any antimicrobial activity *(Table 4)*. The highest antimicrobial activities were observed against *E. faecalis* followed by *S. aureus, L. sakei* ssp. *sakei* and *E. coli* in *L. lactis* ssp. *lactis* isolates obtained from milk samples. *L. lactis* ssp. *lactis* isolated from cheese samples also showed a similar trend exhibiting maximum activity against *E. faecalis (Table 5)*.

Detection of Streptomycin and Tetracycline Resistance Genes by PCR: In 3 isolates obtained from cheese samples, 1 of Tet(S) resistant gene and 2 of Str(A) and Str(B) resistant

Table 3. *Antibiotic resistance rates of L. lactis ssp.lactis isolates obtained from raw sheep cheese and raw milk samples*

Antibiotic	Disk Diffusion Test	Cheese Isolates	Milk Isolates	P
		n (%)	n (%)	
VA[1]	S	30 (100)	30 (100)	-
ST[2]	S	17 (56.7)	18 (60)	1.000
	R	13 (43.3)	12 (40)	
TE[3]	S	26 (86.7)	17 (56.7)	0.020*
	MS	0 (0)	1 (3.3)	
	R	4 (13.3)	12 (40)	
KA[4]	S	30 (100)	30 (100)	-
CN[5]	S	30 (100)	30 (100)	-
ER[6]	S	25 (83.3)	22 (73.3)	0.748
	MS	1 (3.3)	1 (3.3)	
	R	4 (13.3)	7 (23.3)	
DA[7]	S	30 (100)	30 (100)	-
CH[8]	S	30 (100)	30 (100)	-
AM[9]	S	29 (96.7)	24 (80)	0.052
	MS	1 (3.3)	1 (3.3)	
	R	0 (0)	5 (16.7)	

**P<0.05 (S: Sensitive/R: Resistant/MS: Medium-Sensitive)/VA[1]: Vancomycin, ST[2]: Streptomycin, TE[3]: Tetracycline, KA[4]: Kanamycin, CN[5]: Gentamicin, ER[6]: Erythromycin, DA[7]: Clindamycin, CH[8]: Chloramphenicol, AM[9]: Ampicillin*

Table 4. *The inhibitory* activity (%) of kefir *supernatants against selected microorganisms*

Kefir Inhibition	*E. faecalis*	*E. coli*	*S. aureus*	*Listeria*	*B. cereus*
	n (%)	n (%)	n (%)	n (%)	n (%)
No inhibition	30 (100)	9 (30)	27 (90)	26 (86.7)	5 (16.7)
Partial inhibition	0 (0)	17 (56.7)	2 (6.7)	4 (13.3)	15 (50)
High inhibition	0 (0)	4 (13.3)	1 (3.3)	0 (0)	10 (33.3)
Total	30 (100)	30 (100)	30 (100)	30 (100)	30 (100)
P=0.000; P<0.05					

Table 5. *Evaluation of the antimicrobial activity of L. lactis ssp. lactis isolates by agar well diffusion method (The mean value of the inhibition zone in mm)*

Bacteria	Antimicrobial Activity		P
	Cheese Isolates (n=30) Mean±SD	Milk Isolates (n=30) Mean±SD	
E. faecalis	2.95±1.16	2.93±1.29	0.945
E. coli	1.35±1.85	0.59±1.28	0.070
S. aureus	2.15±0.77	2.84±1.1	0.007*
L. sakei	1.74±1.4	0.84±1.52	0.020*
P	0.000*	0.000*	*P<0.05

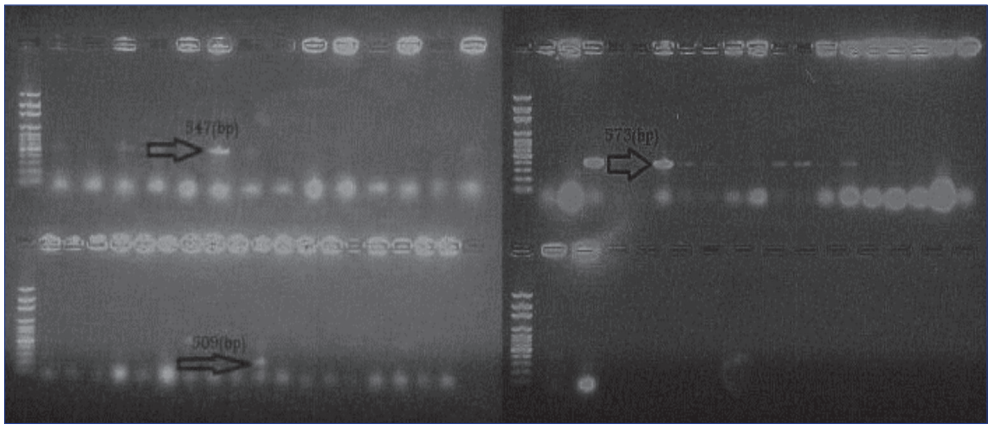

Fig 1. Detection of streptomycin and tetracycline resistance genes in *L. lactis* ssp. *lactis* isolates by PCR test. Left: DNA fragment of 548 and 509 base pairs for Str(A) and Str(B) resistance genes; Right: DNA fragment of 573 base pair for Tet(S) resistance gene

genes were registered. On the other hand in isolates obtained from milk samples, 6 of Tet(S) resistant genes and 5 of Str(A) resistant genes were registered *(Fig. 1)*.

DISCUSSION

During fermentation process, LAB decomposes lactose to produce lactic acid. In this study, the amount of lactic acid production by *L. lactis* ssp. *lactis* isolated from cow milks and cheeses prepared from sheep milk is 0.12±0.07 and 0.14±0.16% respectively. Since the amount of lactose is 4.6% either in cow milk or sheep milk, significant difference was not observed in the amount of lactic acid production. However lactic acid production in kefir supernatants were considerably high (4.31%), as a result of the various types of lactic acid bacteria in kefir. Various species of *Lactobacillus* have been identified and isolated from kefir samples all around the world [24]. These bacteria are among the most important producers of lactic acid. Also the presence of yeast in kefir reduces hydrogen peroxide

and increases the amount of lactic acid. Consequently, it stimulates the growth of other bacteria and the production of kefiran complex [25]. In this manner the importance of using multiple starter culture instead of single starter culture in the dairy industry. Although lactic acid can be produced by chemical methods, the production of lactic acid is much more efficient by starter cultures in terms of flavour and texture of the product. Dimitrellou et al.[26], reported in 2015 that kefir starter cultures exhibited higher proteolytic activity and greater amount of lactic acid production as compared to cheese starter cultures, which was consistent with our study. *Lactococcus. lactis* ssp. *lactis* isolated from different sources, adapted in different environments showed different levels of metabolic activity due to differences in their enzymatic gene expression. *Lactococcus. lactis* ssp. *lactis* isolated from the surface of plants exhibited lower metabolic activity [27]. In this research curd production (curdy appearance) by *L. lactis* ssp. *lactis* isolated from raw milk was higher than cheese isolates. This phenomenon indicated slower metabolic activity of *L. lactis* ssp. *lactis* isolated from cheese. This is an advantage during cheese making in terms of protease enzymes activity, aroma and taste of the product as fast metabolic activity resulted in a bitter taste; rendering *L. lactis* ssp. *lactis* starter culture from cheese an appropriate choice in the cheese making industry. Similar to the results observed in our study, Magalhaes et al.[28] in 2011 examined the extent of pH changes during the fermentation process. According to their results, pH value decreased from 6.61 to 4.42. The decrease in pH indicated the metabolic activity of the microbial population in kefir. By varying the fermentation time, desired taste could be obtained in the kefir drink. The shorter time of fermentation obtained a sweeter kefir quality whereas longer fermentation time resulted in a kefir being more tangy and sour. Chifiriuc et al.[29] analyzed the antimicrobial activity of kefir on pathogenic bacteria. According to their results, the inhibition effect on growth of pathogenic bacteria was observed including *B. subtilis*, *S. aureus*, *E. coli*, *E. faecalis* and *S. enteritidis*. The antimicrobial activity of kefir is due to the production of organic acids including peptides, bacteriocins, ethanol, diacetyl, hydrogen peroxide and carbon dioxide. In addition to inhibiting the growth of pathogenic bacteria, these combinations have been reported to possess a therapeutic effect on the vaginal infections and gastroenteritis [30]. Similar to the results observed in our research, Medrano et al.[31], examined kefir's antagonistic activity against *B. cereus* which is reported to affect the host cell by sphingomyelinase enzyme secretion and its haemolytic activity. Kefir, reduced the hemolytic activity of *B.cereus* by blocking the receptors in the cells and inhibiting sphingomyelinase enzyme. In 2016, Kim et al.[32] found that production of lactic acid and acetic acid by kefir starter cultures prevented the growth of *B. cereus*. Kefir extracts exhibited different antimicrobial activity against different pathogens and displayed probiotic properties [33]. Also, some organic acids in kefir supernatant could inhibit antimicrobial activity of bacteriocins [34,35]. *Enterococcus faecalis* can be both a healthy human gut community member as well as a causative agent of endocarditis, bacteremia, urinary tract infections especially in individuals with suppressed immune system. The specie is often resistant to multiple antibiotics, displaying both inherent and acquired traits [36]. Since *E. faecalis* may be found in natural environment as plants and faeces, it could be observed in raw milk and raw milk cheeses. The *L. lactis* ssp. *lactis* isolated from milk and cheese samples showed the highest antimicrobial activity against *E. faecalis*. In the present study, the highest antimicrobial activity was observed against gram positive and lowest against gram negative bacteria such as *E. coli*. These findings were consistent with findings of Boziaris et al.[37]. In the present study, the highest resistance to chloramphenicol followed by kanamycin and vancomycin antibiotics were observed in kefir samples. These findings were consistent with the findings of Temmerman et al.[38]. According to Danielsen and Wind [39], the sensitivity of *Lactobacillus* species to different antibiotics depended on the species of bacteria and could vary from one strain to another. According to the results of this study, *L. lactis* ssp. *lactis* showed the highest resistance to streptomycin and tetracycline, which were consistent with the results of the study conducted by Devirgilis et al.[40]. In this study, two tetracycline and streptomycin resistance genes were observed in some *L. lactis* ssp. *lactis* isolates obtained from milk and cheese, which were consistent with the results of Zycka-krzesinska et al.[23]. Antibiotic resistance is a major global threat. Tetracycline is an antibiotic that is used in the treatment of mastitis in cows, and as a growth factor to livestock feed. Excessive use of this antibiotic would lead to creating resistant strains. According to Ozpinar, Ondes and Tekiner [41,42], ESBL- and Amp- were the most common phenotypes in Enterobacteriaceae found in foods of animal origin and their encoding genes with a co-resistance pattern, pose a critical foodborne health risk for the consumers. In this respect, excessive and/or unconscious use of antibiotics in farming animals should be taken into consideration and therefore antibiotic use in the veterinary medicine should be intensively monitored. *Lactococcus lactis* ssp. *lactis* strains are widely used in the dairy industry, thus it is necessary to define the properties of this bacterium. The results showed that *L. lactis* ssp. *lactis* bacteria isolated from cheese samples derived from raw sheep milk demonstrated better antimicrobial activity and lactic acid production. On the other hand, antibiotic resistance genes have been detected in some *L. lactis* ssp. *lactis* isolates. Tet(S) resistance gene was confirmed in 7 (11.7%), Str(A) in 6 (10%) and Str(B) in 1 (1.7%) isolates by PCR. Therefore, food can act as a vector. There is a possibility of transferring these genes to *human* commensals and lead to spread resistance genes in microbial *ecosystems*.

Acknowledgment

The authors express acknowledgement to Istanbul Aydin University for its support in this work during in 2016-2017.

REFERENCES

1. Lopitz-Otsoa F, Rementeria A, Elguzabal N, Garaizar J: Kefir: A symbiotic yeast bacteria community with alleged healthy capabilities. *Rev Iberoam Micol*, 23 (2): 67-74, 2000.

2. Marsh AJ, O'Sullivan O, Hill C, Ross RP, Cotter PD: Sequencing-based analysis of the bacterial and fungal composition of kefir grains and milks from multiple sources. *PLoS One*, 8 (7): e69371, 2013. DOI: 10.1371/journal.pone.0069371

3. Otles S, Cagindi O: Kefir: A probitic dairy composition,nutritional and therapeutic aspect. *PJN*, 2, 54-59, 2003. DOI: 10.3923/pjn.2003.54.59

4. Nielsen B, Gürakan GC, Ünlü G: Kefir: A multifaceted fermented dairy product. *Probiotics Antimicrob Proteins,* 6, 123-135, 2014. DOI: 10.1007/s12602-014-9168-0

5. Guzel-Seydim ZB, Kok-Tas T, Greene AK, Seydim AC: Review: Functional properties of kefir. *Crit Rev Food Sci Nutr*, 51, 261-268, 2011. DOI: 10.1080/10408390903579029

6. Ghoneum M, Gimzewski J: Apoptotic effect of a novel kefir product, PFT, on multidrug-resistant myeloid leukemia cells via a hole-piercing mechanism. *Int J Oncol,* 44, 830-837, 2014. DOI: 10.3892/ijo.2014.2258

7. Rodrigues KL, Caputo LRG, Carvalho JCT, Evangelista J, Schneedorf JM: Antimicrobial and healing activity of kefir and kefiran extract. *Int J Antimicrob Agents*, 25 (5): 404-408, 2005. DOI: 10.1016/j.ijantimicag.2004.09.020

8. Dallas DC, Citerne F, Tian T, Silva VLM, Kalanetra KM, Frese SA, Robinson RC, Mills DA, Barile D: Peptidomic analysis reveals proteolytic activity of kefir microorganisms on bovine milk proteins. *Food Chem*, 197, 273-284, 2016. DOI: 10.1016/j.foodchem.2015.10.116

9. Dimitrellou D, Kandylis P, Mallouchos A, Komaitis M, Koutinas AA, Kourkoutas Y: Effect of freeze-dried kefir culture on proteolysis in feta-type and whey-cheeses. *Food Chem,* 119, 795-800, 2010. DOI: 10.1016/j.foodchem.2009.06.052

10. Wassie M, Wassie T: Isolation and identification of lactic acid bacteria from raw cow milk. *Int J Adv Res Biol Sci*, 3 (8): 44-49, 2016.

11. Aziz T, Khan H, Bakhtair SM, Naurin M: Incidence and relative abundance of lactic acid bacteria in raw milk of buffalo, cow and sheep. *J Anim Plant Sci*, 19 (4): 168-173, 2009.

12. Hladíková Z, Smetanková J, Greif G, Greifová M: Antimicrobial activity of selected lactic acid cocci and production of organic acids. *Acta Chim Slov*, 5 (1): 80-85, 2012. DOI: 10.2478/v10188-012-0013-3

13. Kim SY, Kim JE, Lee KW, Lee HJ: *Lactococcus lactis* ssp. *lactis* inhibits the proliferation of SNU-1 human stomach cancer cells through induction of G0/G1 cell cycle arrest and apoptosis via p53 and p21 expression. *Ann N Y Acad Sci*, 1171, 270-275, 2009. DOI: 10.1111/j.1749-6632.2009.04721.x

14. Rodríguez-Figueroa JC, González-Córdova AF, Astiazaran-García H, Vallejo-Cordoba B: Hypotensive and heart rate-lowering effects in rats receiving milk fermented by specific *Lactococcus lactis* strains. *Br J Nutr*, 109, 827-833, 2013. DOI: 10.1017/S0007114512002115

15. Rattanachaikunsopon P, Phumkhachorn P: Lactic acid bacteria: their antimicrobial compounds and their uses in food production. *Ann Biol Res,* 1, 218-228, 2010.

16. Guessas B, Kihal M: Characterization of lactic acid bacteria isolated from Algerian arid zone raw goats' milk. *Afr J Biotechnol*, 3 (6): 339-342, 2004. DOI: 10.5897/AJB2004.000-2062

17. Khemariya P, Singh S, Nath G, Gulati AK: Isolation,identification,and antibiotic susceptibility of nis+ *Lactococcus lactis* from dairy and non-dairy sources. *Czech J Food Sci*, 31, 323-331, 2013.

18. Buyukyoruk S, Cibik R, Cetinkaya F, Soyutemiz GE, Goksoy EO, Kirkan S: Isolation,phenotypic and molecular identification of *Lactococcus lactis* isolation from traditionally produced village cheeses. *Asian J Anim Vet Adv*, 9 (16): 2154-2158, 2010. DOI: 10.3923/javaa.2010.2154.2158

19. Facklam R, Elliott JA: Identification, classification, and clinical relevance of catalase-negative,gram-positive cocci, excluding the streptococci and enterococci. *Clin Microbiol Rev*, 8 (4): 479-495, 1995.

20. Atanasova J, Moncheva P, Ivanova I: Proteolytic and antimicrobial activity of lactic acid bacteria grown in goat milk. *Biotechnol Biotechnol Equip*, 28 (6): 1073-1078, 2014. DOI: 10.1080/13102818.2014.971487

21. Clinical and Laboratory Standards Institute: Performance standards for antimicrobial disk susceptibility test; approved standard. *CLSI document M02-A11*, 32 (1): 2012**.**

22. Klare I, Konstabel C, Werner G, Huys G, Vankerckhoven V, Kahlmeter G, Hildebrandt B, Muller-Bertling S, Witte W, Goossens H: Antimicrobial susceptibilities of *Lactobacillus, Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. *J Antimicrob Chemother*, 59, 900-912, 2007. DOI: 10.1093/jac/dkm035

23. Zycka-Krzesinska J, Boguslawska J, Aleksandrzak-Piekarczyk T, Jopek J, Bardowski JK: Identification and characterization of tetracycline resistance in *Lactococcus lactis* isolated from Polish raw milk and fermented artisanal products. *Int J Food Microbiol*, 211, 134-141, 2015. DOI: 10.1016/j.ijfoodmicro.2015.07.009

24. Zhou J, Liu X, Jiang H, Dong M: Analysis of the microflora in Tibetan kefir grains using denaturing gradient gel electrophoresis. *Food Microbiol*, 26, 770-775, 2009. DOI: 10.1016/j.fm.2009.04.009

25. Cheirsilp B, Shimizu H, Shioya S: Enhanced kefiran production by mixed culture of *Lactobacillus kefiranofaciens* and *Saccharomyces cerevisiae*. *J Biotechnol*, 100, 43-53, 2003. DOI: 10.1016/S0168-1656(02)00228-6

26. Dimitrellou D, Kandylis P, Kourkoutas Y, Koutinas AA, Kanellaki M: Cheese production using kefir culture entrapped in milk proteins . *Appl Biochem Biotechnol*, 176 (1): 213-230, 2015. DOI: 10.1007/s12010-015-1568-4

27. Klijn N, Weerkamp AH, de Vos WM: Detection and characterization of lactose-utilizing *Lactococcus* spp. in natural ecosystems. *Appl Environ Microbiol*, 61 (2): 788-792, 1995.

28. Magalhães KT, de Melo Pereira GV, Campos CR, Dragone G, Schwan RF: Brazilian kefir: Structure,microbial communities and chemical composition. *Braz J Microbiol*, 42, 693-702, 2011. DOI: 10.1590/S1517-83822011000200034

29. **Chifiriuc MC, Cioaca AB, Lazar V:** *In vitro* assay of the antimicrobial activity of kephir against bacterial and fungal strains. *Anaerobe*, 17, 433-435, 2011. DOI: 10.1016/j.anaerobe.2011.04.020

30. Sarkar S: Biotechnological innovations in kefir production: a review. *Brit Food J,* 110 (3): 283-295, 2008. DOI: 10.1108/00070700810858691

31. Medrano M, Fernando Perez P, Abraham AG: Kefiran antagonizes cytopathic effects of *Bacillus cereus* extracellular factors. *Int J Food Microbiol*, 122, 1-7, 2008. DOI: 10.1016/j.ijfoodmicro.2007.11.046

32. Kim DH, Jeong D, Kim H, Kang IB, Chon JW, Song KY, Seo KH: Antimicrobial activity of kefir against various food pathogens and spoilage bacteria. *Korean J Food Sci Anim Resour,* 36 (6): 787-790, 2016. DOI: 10.5851/kosfa.2016.36.6.787

33. Kim DH, Chon JW, Kang IB, Kim H, Kim HS, Song KY, Seo KH: Growth inhibition of *Cronobacter sakazakii* in experimentally contaminated powdered infant formula by kefir supernatant. *J Food Prot,* 78, 1651-1655, 2015. DOI: 10.4315/0362-028X.JFP-15-119

34. Joshi VK, Sharma S, Rana NS: Production, purification, stability and efficacy of bacteriocin from isolates of natural lactic acid fermentation of vegetables. *Food Technol Biotechnol*, 44, 435-439, 2006.

35. Dogan M, Ozpınar H: Investigation of probiotic features of bacteria isolated from some food products. *Kafkas Univ Vet Fak Derg*, 23 (4): 555-562, 2017. DOI: 10.9775/kvfd.2016.17273

36. Arias CA, Murray BE: The rise of the *Enterococcus*: Beyond vancomycin resistance. *Nat Rev Microbiol*, 10, 266-278, 2012. DOI: 10.1038/nrmicro2761

37. Boziaris IS, Adams MR: Transient sensitivity to nisin in cold shocked Gram negatives. *Lett Appl Microbiol*, 31, 233-237, 2000. DOI: 10.1046/j.1365-2672.2000.00804.x

38. Temmerman R, Pot B, Huys G, Swings J: Identification and antibiotic susceptibility of bacterial isolates from probiotic products. *Int J Food Microbiol*, 81, 1-10, 2003. DOI: 10.1016/S0168-1605(02)00162-9

39. Danielsen M, Wind A: Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *Int J Food Microbiol*, 82, 1-11, 2003. DOI: 10.1016/S0168-1605(02)00254-4

40. Devirgilis C, Barile S, Caravelli A, Coppola D, Perozzi G: Identification of tetracycline and erythromycin-resistant Gram-positive cocci within the fermenting microflora of an Italian dairy food product. *J Appl Microbiol*, 109, 313-323, 2010. DOI: 10.1111/j.1365-2672.2010.04661.x

41. Tekiner IH, Ozpinar H: Occurrence and characteristics of extended spectrum beta-lactamases-producing Enterobacteriaceae from foods of animal origin. *Braz J Microbiol,* 47, 444-451, 2016. DOI: 10.1016/j.bjm.2015.11.034

42. Ondes N, Ozpinar H: Occurrence of ESBL-producing enterobacteriaceae in red meat samples. *Kafkas Univ Vet Fak Derg*, 22 (1): 79-83, 2016. DOI: 10.9775/kvfd.2015.13944