Antibiotic Resistance Patterns of Lactic Acid Bacteria Isolated From Different Fermented Milk Products of Turkish Origin

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Authors' contributions

This work was carried out in collaboration between both authors. Author GB designed the study, wrote the protocol and wrote the first draft of the manuscript. Author MO managed the analyses of the study. Both authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aims: Lactic acid bacteria isolated from 48 Turkish fermented milk products obtained from local markets, dairies or bazaars were investigated for their resistance of antibiotics including ampicillin, erythromycin, gentamicin, chloramphenicol, lincomycin, meropenem, ciprofloxacin, teicoplanin, tetracycline and vancomycin.
Place and Duration of Study: Adnan Menderes University Biology Department, Microbiology laboratory, between 2012-2014
Methods: LAB strains, belonging to 14 species of *Lactobacillus* (n=68), 1 species of *Lactococcus* (n=16), 5 species of *Enterococcus* (n=14) and 2 species of *Streptococcus* (n=17) were isolated and identified at species level by their 16S rRNA gene sequencing. Minimal Inhibitory Concentrations (MIC) for 10 antibiotics were determined by agar dilution test using multipoint inoculator. Antibiotic resistance genes for erythromycin [*erm*(A), *erm* (B), *erm* (C)], gentamycin *aac(6') aph(2'')*, chloramphenicol (*cat*), tetracycline [*tet*(K), *tet*(L), *tet*(M), *tet*(S), *tet*(Q)] and vancomycin [*van*(A), *van*(B), *van*(C), *van*(X)] were investigated in strains. Mating experiments were done with *E. faecalis* JH2-2 to detect the transferability of resistance genes.

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Results: Among 115 strains antibiotic resistance was detected against lincomycin (27,8%), tetracycline (20%), ampicillin (13,9%), meropenem (11,3%), gentamycin (10,4%), erythromycin (7,8%), ciprofloxacin (6,1%), chloramphenicol (3,4%), vancomycin (0,87%). While all these strains were susceptible to teicoplanin, 29,5% of isolates were multiple resistant to various antibiotics. The resistance genes *aac*(6') *aph*(2''), *erm*(B), *tet*(L), *tet*(M) and *van*(C) were detected in strains of, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. kefiri*, *Lc. lactis* subsp. *lactis*, *S. lutetiensis*, *S. macedonicus*, *E. faecalis, E. gallinarum* isolated from some cheeses and one household kefir samples.
Conclusion: Antibiotic-resistant LAB carrying transeferable resistance genes in some Turkish dairy products, may act as a dangerous vehicle for transmission of these traits to the other bacteria by horizontal gene transfer.

1. INTRODUCTION

Lactic acid bacteria (LAB) have a long history of safe use as fermenting natural products and have acquired the "Generally recognized as safe" (GRAS) status [1]. Many LAB species are present in fermented foods as contaminants or deliberately added as starter culture for preparation and preservation purposes [2]. They are beneficial for human physiology, specifically digestion and preventing microbial disorders as natural intestinal microflora of humans [3].

Although antibiotic resistance (AR) in clinically relevant bacteria known for a long time, studies on those belonging to LAB groups increased in recent years because they can serve as reservoir for antibiotic resistance genes and transfer them to the other microorganisms including pathogens [4]. The extensive use of antibiotics to non-human applications (feed, agriculture and veterinary applications) has exerted a very strong selective pressure resulting in the appearance of resistant strains.

Food chain, especially fermented products that are not heat treated before consumption has been considered as the main route for the introduction of AR bacteria into the gastrointestinal tract (GI) [5]. When carried on mobile genetic elements such as conjugative transposons or plasmids, AR traits can potentially be transferred to the human commensal flora or pathogenic bacteria in the hosts [6]. European Food Safety Authority (EFSA) recommends that bacterial strains carrying transferable antibiotic resistance genes should not be used in animal feeds, fermented and probiotic foods for human [7]. EFSA has also proposed and updated "microbiological breakpoints" for several genera of LAB in order to check for transferable AR signs in starter cultures.

The aim of the present study was to determine the phenotypic and genotypic antibiotic resistance in lactic acid bacteria isolated from fermented dairy products in Aydin, Turkey.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Lab

Bacterial strains were isolated from fermented dairy, homemade-bazaar and supermarketed food samples including white-cheese (n=9), çökelek-cheese (n=2), kefir (n=4), sheep-cheese (n=6), lor (Turkish whey cheese) (n=6), tulum cheese (n=9), yoğurt (n=12). Food samples were homogenized and serial dilutions were plated on MRS agar plates supplemented with cycloheximide (50 mg/L) and incubated at 35℃ Gas generating System, Sigma) [8]. After the incubation, colonies were purified twice and then isolates stored at -20℃ in MRS broth with 10% glycerol. Bacterial isolates were activated in MRS broth prior to all experiments.

All isolated strains were preliminary identified with their phenotypic features. For this purpose Gram staining, catalase test and colony morphologies were evaluated. Strain identification also done according to 16S rDNA sequence analysis and BLAST in GenBank database (www.ncbi.nlm.nih.gov).

2.2 Antibiotic Susceptibility Testing

Minimal Inhibitory Concentrations (MIC) for 10 antibiotics were determined by agar dilution test using multipoint inoculator. Isolates were grown in MRS broth (Merck, Darmstadt, Germany) for 48 hours and then inoculated to LSM Agar (90% Iso-Sensitest[TM] Broth (Oxoid) + 10% MRS Broth (Merck)+ 1,5% Agar (Merck) [9] plates containing ampicillin, erythromycin, gentamicin,

chloramphenicol, lincomycin, meropenem, ciprofloxacin, teicoplanin, tetracycline and vancomycin antibiotics (Oxoid, Hampshire, UK) with the concentration range of 0.0625-128 µg/ml or 0.0625-64 µg/ml for ciprofloxacin. The MIC was defined as the lowest concentration of antibiotic giving a complete inhibiton of visible growth in comparison to an antibiotic free control point. Breakpoints were adopted from [10,11,12] (Table 1).

2.3 Amplification of Antibiotic Resistance Genes

Antibiotic resistance genes for erythromycin [*erm*(A), *erm* (B), *erm* (C)], gentamycin *aac(6')* *aph(2'')*, chloramphenicol (*cat*), tetracycline [*tet*(K), *tet*(L), *tet*(M), *tet*(S), *tet*(Q)] and vancomycin [*van*(A), *van*(B), *van*(C), *van*(X)] were investigated in all strains except for LAB groups which are intrinsically resistant to vancomycin. Primers used for amplification of resistance genes are given in Table 2. The reaction mixtures (25 µL) contained 20 pmol of each primer, 2.0 mmol $MgCl_2$, 200 µmol dNTP, 20 ng/ µL bacterial DNA and 1 U of Taq polymerase. DNA fragment sizes and annealing temperatures summarized in Table 2. Positive controls were used for *erm*(A), *erm* (B), *erm* (C), *aac(6') aph(2'')*, *tet*(M), *van*(A), *van*(C), *van*(X) genes. PCR products were checked electrophoretically on 1,5% agarose gel prepared with SafeView (5%, w/v). Amplicons were sequenced, and then gene sequences obtained were analyzed using the BLAST (blastn) search programme.

2.4 Mating Experiments

E. faecalis JH2-2 (resistant to rifampin and fusidic acid) was used as recipient in mating experiments. Agar plates prepared for selection of transconjugants contained fusidic acid (20 µg/mL) with rifampin (50 µg/mL) combined with erythromycin (20 µg/mL), gentamicin (100 µg/mL), tetracycline (20 µg/mL) according to which resistance gene's transferability was tested [13]. The *tet*(L), *tet*(M), *erm*(B), *aac*(6') *aph*(2'') positive strains isolated in this study were used as donors. One mL of donor and 1 mL of recipient strain, at exponential growth were mixed, filtered through a sterile 0.45 µm-pore-size nitrocellulose membrane filter (Millipore, USA), and placed on MRS or BHI agar plates. After incubation at 37℃ for 24 h, cells were resuspended in sterile saline and were spread on

antibiotics contained selective medium [4]. Following incubation at 37℃ for 24 to 72 h, plates were checked for the absence (donor and recipient plates) or presence (mating mixture plate) of growth and conjugation frequencies were estimated.

3. RESULTS

3.1 Isolation and İdentification of LAB Strains

Totally one hundred-fifteen LAB strains were isolated on MRS media from 48 fermented milk products. As a result of BLAST analysis of 16S rDNA sequences, isolates belonging to species of *Lb. helveticus* (n=2), *Lb. acidophilus* (3), *Lb. delbrueckii* (28), *Lb. gasseri* (1), *Lb. uvarum* (1), *Lb. brevis* (1), *Lb. coryniformis* (1), *Lb.curvatus* (5), *Lb. kefiri* (8), *Lb. alimentarius*

(2), *Lb. diolivorans* (1), *Lb. otakiensis* (1), *Lb. rhamnosus* (9), *Lb. paracasei* (5), *E. faecalis* (5), *E. durans* (3), *E. faecium* (3), *E. gallinarium* (2), *E. hirae* (1), *Lc. lactis subsp. lactis* (16), *S. macedonicus* (11), *S. lutitiensis* (6).

The most part of strains were isolated from lor cheeses (25,6%), followed by those from tulum cheeses (22%), white cheeses (16,10%), yoghurts (12,5%), kefirs (12,5%) and sheep cheeses (11,3%).

3.2 Phenotypic Profile of Antimicrobial Resistances

Totally one hundred-fifteen LAB strains isolated on MRS media from 48 fermented milk products were tested by agar dilution method. Table 3. shows the number of LAB species tested for antibiotic susceptibility, MIC value ranges and number of resistant strains for each species. Antibiotic resistance patterns were different depending on LAB species except for teicoplanin that all strains were susceptible.

When considered all the strains antibiotic resistance levels were as follows; lincomycin (27,82%), tetracyclin (20%), ampicillin (13,91%), meropenem (11,30%), gentamycin (10,43%), erythromycin (7,82%), ciprofloxacin (6,08%), chloramphenicol (3,46%), vancomycin (0,87%). Streptococci and Enterococci seemed like the most resistance genera to the tested antibiotics and multiple drug resistance was present in seven isolate (50%) of Enterococci, and seven of

(41,17%) Streptococci. While 23,52% of Lactobacilli strains were multiple resistant, this trait was not detected in any Lactococci (data not shown).

Table 1. Breakpoints proposed for different LAB groups

LAB group	MIC breakpoints (µg/mL)									
	Ampicillin	Erytromycin	Gentamycin	Chloramphenicol	Lincomycin	Meropenem	Ciprofloxacin	Teicoplanin	Tetracyclin	Vancomycin
Lactobacillus obligate homofermentative	1[1]	1[1]	16[1]	4[1]	1[1]	8[3]	32[2]	8[3]	4[1]	2[1]
Lactobacillus obligate heterofermentative	2[1]	1[1]	16[1]	4[1]	1[1]	8[3]	32[2]	IR	8[1]	IR
Lactobacillus facultative heterofermentative	4[1]	1[1]	16[1]	4[1]	1[1]	8[3]	32[2]	IR	8[1]	IR
Enterococci	4[1]	4[1]	32[1]	8[1]	4[1]	8[4]	2[5], 4[5*]	8[4]	2[1]	4[1]
Streptococcus	2[1]	2[1]	32[1]	4[1]	2[1]	8[4]	32[2]	8[4]	4[1]	4[1]
Lactococci	2[1]	2[1]	32[1]	8[1]	4[1]	8[4]	32[2]	8[4]	4[1]	4[1]

1, EFSA 2008 [10]; 2, Danielsen and Wind (2003) [12]; 3, Daimmo (2007) [14]; 4, Walsh (2003) [15]; 5, European commission (2003 [16]); *breakpoint for E. Faecium

Table 2. Primers, annealing temperatures and expected sizes for PCR reactions in this study

Target gene	Primer sequence (5'→3')	T_a ∘C	Fragment size (bp)	Reference
erm (A)	ermA1:TCTAAAAAGCATGTAAAAGAA ermA2:CTTCGATAGTTTATTAATATTAGT	52	645	[17]
erm (B)	ermB1: GAAAAGGTACTCAACCAAATA ermB2: AGTAACCGTACTTAAATTGTTTAC	52	639	[17]
erm (C)	ermC1: TCAAAACATAATATAGATAAA ermC2: GCTAATATTGTTTAAATCGTCAAT	52	642	[17]
aac(6') *aph*(2'')	aac(6') aph(2'') F:CCAAGAGCAATAAGGGCATA aac(6') aph(2'') R: CACTATCATAACCACTACCG	60	220	[18]
Cat	cat-TC F: CATATCAAATGAACTTTAATA cat-TC R: CGTTTTGTGAAGTAGTACACT	52	718	[19]
tet (K)	tetKI: CAATACCTACGATATCTA tetKII: TTGAGCTGTCTTGGTTCA	50	352	[9]
tet (L)	tetLI: TGGTCCTATCTTCTACTCATTC tetLII: TTCCGATTTCGGCAGTAC	54	385	[20]
tet (M)	tetMI: GGTGAACATCATAGACACGC tetMII: CTTGTTCGAGTTCCAATGC	52	401	[20]
tet (S)	tetS-FW: ATCAAGATATTAAGGAC tetS-RV: TTCTCTATGTGGTAATC	55	573	[21]
tet (Q)	tetQ-FW: AGAATCTGCTGTTTGCCAGTG tetQ-RV: CGGAGTGTCAATGATATTGCA	63	169	[22]
van (A)	vanA-36F: TTGCTCAGAGGAGCATGACG vanA-992R: TCGGGAAGTGCAATACCTGC	65	957	[22]
van (B)	vanB-23F: TTATCTTCGGCGGTTGCTCG vanB-1016R: GCCAATGTAATCAGGCTGTC	62	994	[23]
van (C)	vanC-F: CAGTGTCACTAACCTCAGCAGCCG vanC-R: TAGGATAACCCGACTTCCGCCA	64	934	[24]
van (X)	vanXSACF: CACTTCCCGAGCTCATTGACCGCTTGATCG vanXKPNR: CCGAAAGAGGTACCTTATATAGTTTGTCCG	60	740	[24]

Table 3. MIC value ranges and number of resistant strains

Bacterial species (n[a])	Antibiotic MIC range (µg/mL) (n[b])									
	Ampicillin		Erythromycin		Gentamycin		Chloramphenicol		Lincomycin	
Lb. helveticus (2)	≤0,0625-0,5		≤0,0625		4		2		0,125-2	(1)
Lb. acidophilus (3)	2	(3)	≤0,0625		2-4		2		0,5-1	
Lb.delbrueckii (28)	≤0,0625-4	(5)	≤0,0625-1		≤0,0625-64	(1)	≤0,0625-8	(1)	≤0,0625-32	(6)
Lb. gasseri (1)	1		≤0,0625		16		4		2	(1)
Lb. uvarum (1)	2	(1)	≤0,0625		0,125		2		≤0,0625	
Lb. brevis (1)	8	(1)	0,5		4		4		8	(1)
Lb. coryniformis subsp. Torguens (1)	2		0,125		2		4		0,5	
Lb. curvatus (5)	0,5-4	(1)	≤0,0625-0,125		0,125-16		1-4		≤0,0625-1	
Lb. kefiri (8)	0,5-2		≤0,0625- ≥128	(1)	≤0,0625-0,5		1-4		≤0,0625-≥128	(4)
Lb. alimentarius (2)	4		≤0,0625		1-2		2-4		2	(2)
Lb. diolivorans (1)	0,5		≤0,0625		0,25		1		≤0,0625	
Lb. otakiensis (1)	4		≤0,0625		0,125		2		≤0,0625	
Lb. rhamnosus (9)	≤0,0625-8	(1)	≤0,0625-0,25		0,5-8		2-8	(3)	≤0,0625-2	(4)
Lb. paracasei (5)	2-8	(3)	≤0,0625-0,25		0,25-16		1-4		≤0,0625-2	
E. faecalis (5)	2-4		0,25-1		32-≥128	(3)	4-8		16-≥128	(5)
E. durans (3)	1-2		≤0,0625-0,125		2-8		2-8		≤0,0625-1	
E. faecium (3)	1-4		2		8-32		4		0,5-32	(1)
E. gallinarium (2)	4		0,25-0,5		8		4		≤0,0625-32	(1)
E. hirae (1)	0,5		32	(1)	16		8		64	(1)
Lc. Lactis subsp. Lactis (16)	0,25-4	(1)	≤0,0625-0,125		≤0,0625-≥128	(1)	1-4		≤0,0625-2	
S. macedonicus (11)	0,5-1		≤0,0625-≥128	(7)	0,5-≥128	(6)	1-4		≤0,0625-16	(4)
S. lutetiensis (6)	0,25-1		≤0,0625		0,25-64	(1)	1-4		≤0,0625-4	(1)
Total	16 (13,91%)		9 (7,82%)		12 (10,43%)		4 (3,47%)		32 (27,82%)	

n[a], number of isolated strains; n[b], number of resistant strains

Table 3. Continued

Bacterial species (n[a])	Antibiotic MIC range (µg/mL) (n[b])								
	Meropenem		Ciprofloxacin		Teicoplanin	Tetracycline		Vancomycin	
Lb. helveticus (2)	0,25		16-32		0,125	1		0,5	
Lb. acidophilus (3)	0,25-0,5		16-32		≤0,0625-0,125	1		0,25	
Lb.delbrueckii (28)	≤0,0625-0,25		≤0,0625-16		≤0,0625-0,5	0,125-32	(2)	0,125-0,5	
Lb. gasseri (1)	1		64	(1)	0,25	2		1	
Lb. uvarum (1)	0,5		0,5		4	0,5		≥128	(1)
Lb. brevis (1)	0,5		32		≥128	16	(1)	≥128	
Lb. coryniformis subsp. Torguens (1)	2		8		≥128	32	(1)	≥128	
Lb.curvatus (5)	≤0,0625-2		0,5-8		32-≥128	0,5-32	(1)	≥128	
Lb.kefiri (8)	≤0,0625-0,125		1-16		8-≥128	16-32	(8)	≥128	
Lb. alimentarius (2)	1		4-16		64-≥128	1-8		≥128	
Lb. diolivorans (1)	≤0,0625		1		32	1		0,5	
Lb.otakiensis (1)	0,25		8		≥128	16	(1)	≥128	
Lb. rhamnosus (9)	0,5-16	(8)	0,5-2		≥128	1-2		≥128	
Lb. paracasei (5)	1-8	(1)	0,5-4		32-≥128	0,25-1		≥128	
E.faecalis (5)	2-8	(1)	0,5-4	(4)	≤0,0625	0,5-32	(1)	0,5-2	
E.durans (3)	0,125-16	(2)	0,5-2		≤0,0625-0,125	0,25-2		0,25-0,5	
E. faecium (3)	0,5-16	(1)	1-4	(2)	0,125-0,25	0,25-1		0,5-1	
E.gallinarium (2)	2		2		≤0,0625-0,125	0,25-0,5		8	
E. hirae (1)	1		1		0,25	0,5		0,125	
Lc. Lactis subsp. Lactis (16)	≤0,0625		1-4		≤0,0625	≤0,0625-0,5		0,125-0,5	
S. macedonicus (11)	≤0,0625		0,5-4		≤0,0625	0,25-≥128	(7)	0,125-0,5	
S.lutetiensis (6)	≤0,0625		0,25-2		≤0,0625-0,125	≤0,0625-32	(1)	≤0,0625-0,25	
Total	11 (11,30%)		7 (6,08%)		0	23 (20%)		1 (0,87%)	

n[a], number of isolated strains; n[b], number of resistant strains

Table 4. Antibotic resistance genes, MIC value of related antibiotic and origins of LAB isolates

Resistance gene/genes	MIC value	Isolate	Bacterial species	Origin
tet(L)	32	GLM185	*Lb. delbrueckii* subsp. *bulgaricus*	Tulum cheese (dairy)
erm(B)	≥128	GLM 76	*Lb. kefiri*	Kefir (household)
tet(M)	32	GLM 77	*Lb. kefiri*	Kefir (household)
aac (6') *aph* (2'')	≥128	GLM 152	*Lc. lactis* subsp. *lactis*	White cheese (dairy)
aac (6') *aph* (2'')	32	GLM 112	*Lc. lactis* subsp. *lactis*	Lor cheese (dairy)
tet(M)	32	GLM 116	*S. lutetiensis*	Lor cheese (dairy)
aac (6') *aph* (2'')	≥128	GLM 151	*S. macedonicus*	White cheese (dairy)
erm(B)	≥128			
tet(L)	32			
tet(M)				
aac (6') *aph* (2'')	4	GLM 198	*S. macedonicus*	Tulum cheese (dairy)
erm(B)	≥128			
tet(M)	64			
aac (6') *aph* (2'')	≥128	GLM 146	*S. macedonicus*	White cheese (dairy)
erm(B)	≥128			
tet(M)	32			
aac (6') *aph* (2'')	≥128	GLM 187	*S. macedonicus*	Tulum cheese (dairy)
*erm(*B)	≥128			
tet(M)	≥128			
aac (6') *aph* (2'')	≥128	GLM 193	*S. macedonicus*	Tulum cheese (dairy)
erm(B)	≥128			
tet(M)	64			
aac (6') *aph* (2'')	≥128	GLM 206	*S. macedonicus*	Sheep cheese (dairy)
erm(B)	≥128			
tet(L)	64			
tet(M)				
aac (6') *aph* (2'')	≥128	GLM 207	*S. macedonicus*	Sheep cheese (dairy)
erm(B)	≥128			
tet(L)	64			
tet(M)				
aac (6') *aph* (2'')	≥128	GLM 132	*E. faecalis*	White cheese (market)
tet (M)	32	GLM 183	*E. faecalis*	Tulum cheese (dairy)
van(C)	8	GLM 129	*E. gallinarum*	White cheese (dairy)
van(C)	8	GLM 157	*E. gallinarum*	White cheese (market)

3.3 Antibiotic Resistance Genes

Antibiotic resistance genes detected by PCR, isolates and source of samples are summarized in Table 4. The *aac*(6') *aph*(2''), *erm*(B), *tet*(L), *tet*(M) and *van*(C) genes were detected in seventeen LAB isolates obtained from various cheeses and household kefir samples. Gentamycin resistance gene *aac*(6') *aph*(2'') was found in ten LAB strains including *Lc. lactis subsp. Lactis* (2), *S. macedonicus* (7) and *E. faecalis* (1) All of these isolates showed MIC of gentamycin ≥128 µg/mL, except for *S. macedonicus* GLM-198 showed MIC of 4 µg/mL. Sequence analyses of *aac*(6') *aph*(2'') genes resulted in maximum identity with the transposons such as TN*6218* (Accession number HG002387.1) and Tn*4001* (Accession number AB682805.1) of pathogenic bacteria like *Clostridium difficile* and *S. aureus*. The *tet*(L) gene was detected in the 4 *Lb. delbrueckii* subsp. *bulgaricus* and 3 *S. macedonicus* strains with tetracycline MICs of 32-64 µg/mL. In addition, another tetracycline resistance gene *tet*(M) amplified from *Lb. kefiri* (1), *S. lutetiensis* (1) ve *S. macedonicus* (7) and *E. faecalis* (1) strains with MICs of tetracycline 32-≥128 µg/mL. Amplicons showed 99-100% identity with *tet*(M) sequences of Tn*5801* and Tn*4011* transposons of *E.faecium* (Accession number KP001176.1 and KP036966.1). Isolates which possess *erm*(B) gene were *S. macedonicus* (7), *Lb. kefiri* (1), showed MIC of erythromycin ≥128 µg/mL. The sequences of the *erm*(B) genes amplified from our isolates proved to be 99-100% identical to the sequences in the Tn6194 transposon of *Clostridium difficile* (HG475346.1).

We also investigated the mobility of the detected *tet*, *erm* and *aac(6') aph(2'')* genes in filter mating experiments with recipient strain *E. faecalis* JH2-2. Transconjugant colonies were obtained from tested donors *S. macedonicus* 193 and *S. macedonicus* 207 in rifampin-fusidic acid (RF)-gentamicin and rifampin-fusidic acid- gentamycin, RF-tetracycline and RF-erythromycin plates respectively. Transfer frequencies obtained during filter matings were in the range of 10^5 to 10^6 per recipient.

4. DISCUSSION

Dairy products like yogurt and cheese are largely consumed foods in Turkey. It is very important to determine the LAB in fermented products and antibiotic resistance profiles of these bacteria, also compare the products according to their sources and qualifications. Lactic starter cultures used in fermented milk products and these bacteria enter into human gastrointestinal tract in large numbers and they can transfer resistance genes to intestinal pathogens.

In our study, the most frequently seen resistances of LAB are lincomycin (27,8%), tetracycline (20%) and ampicillin (13,9%), respectively and all LAB isolates were susceptible to teicoplanin. When considered sources where the foods obtained from, highest percentage of antibiotic resistant bacteria was found in dairy isolates (66,6%), followed by those obtained from local markets (41,02%) and bazaars (20%) (Fig. 1.). The extent of contamination with antimicrobial-resistant LAB was lower in yogurt samples than in cheeses. Among all the nineteen lactobacilli isolated from yogurt samples, only GLM 49 (resistant to lincomycin and tetracyclin) and GLM 56 (resistant to ampicillin and meropenem) were antibiotic resistant (Table 5).

Among Lactobacilli resistance levels were as follows; lincomycin (29,4%), ampicillin (22%) ve tetracycline (20,6%), meropenem (13,2%), chloramphenicol (5,8%), erythromycin (1,4%), gentamycin (1,4%), ciprofloxacin (1,4%), vancomycin (1,4%). Lactobacilli strains belonging to obligate heterofermentative and facultative heterofermentative groups e.g. *Lb. brevis*, *Lb. coryiniformis*, *Lb. curvatus*, *Lb. kefiri, Lb. alimentarius*, *Lb. otakiensis*, *Lb. rhamnosus, Lb. paracasei* isolated in our study were resistant to vancomycin supporting to natural resistance of these groups to vancomycin [12]. The vancomycin resistance in these species is intrinsic due to their possesion of D-Ala-D-Lactate in their peptidoglycan rather than the D-Ala-D-Ala dipeptide. Only one isolate, *Lb. uvarum* GLM 101 which is belonging to homofermentative group, was resistant to vancomycin. While tetracycline resistant two strains *Lb. delbrueckii* subsp. *bulgaricus* GLM 185 and *Lb. kefiri* GLM 77 harboured *tet*(L) and *tet*(M) genes respectively, *erm*(B) gene was detected in *Lb. kefiri* GLM76 (MIC of erythromycin ≥128 µg/mL). Tet genes were identified from *Lb. kefiri* NWI78 isolated from probiotic yogurt [4]. While *tet*(M) gene is ribosomal protection gene carried by Tn916 or related conjugative transposon and is typically located on chromosome *tet*(L) genes encode efflux proteins and carry out on plasmids [25]. Both of tetracycline resistance genes detected in many lactobacilli species [19,11,4]. *erm*(B) gene

is responsible for a posttranscriptional methylase-mediated modification of the 23S rRNA and is one of the most common erythromycin resistance gene in lactobacilli [19,11,26]. In general, two of the most commonly observed resistance genes in LAB found so far are *tet*(M)-for tetracycline resistance and *erm*(B)-for erythromycin, followed with *cat* genes coding for chloramphenicol resistance [27].

Lc. lactis subsp. *lactis* and *Lc. Lactis* subsp. *cremoris* are technologically important lactococci species and among the sixteen *Lc. lactis* subsp. *lactis* strains isolated in the study, one strain was resistant to ampicilin (with MIC value of 4 µg/mL)

and gentamycin resistance gene *aac* (6) *aph* (2'') was found in two strains, GLM 112 and GLM 152 with MIC value of 32 µg/mL and 128 µg/mL respectively. No other phenotypic or genotypic antibiotic resistance was determined in Lactococci and it is possible to say that they were the most susceptible LAB group in our study. *Lc. lactis* have been reported as usually susceptible to the macrolides, bacitracin, erythromycin, lincomycin, novobiocin, teicoplanin, vancomycin, rifampicin, spectinomycin, chloramphenicol, penicillin and ampicilin [2]. *Lc. lactis* subsp. *lactis* K214 isolated from raw milk cheese has at least three plasmid conferring resistance to tetracycline,
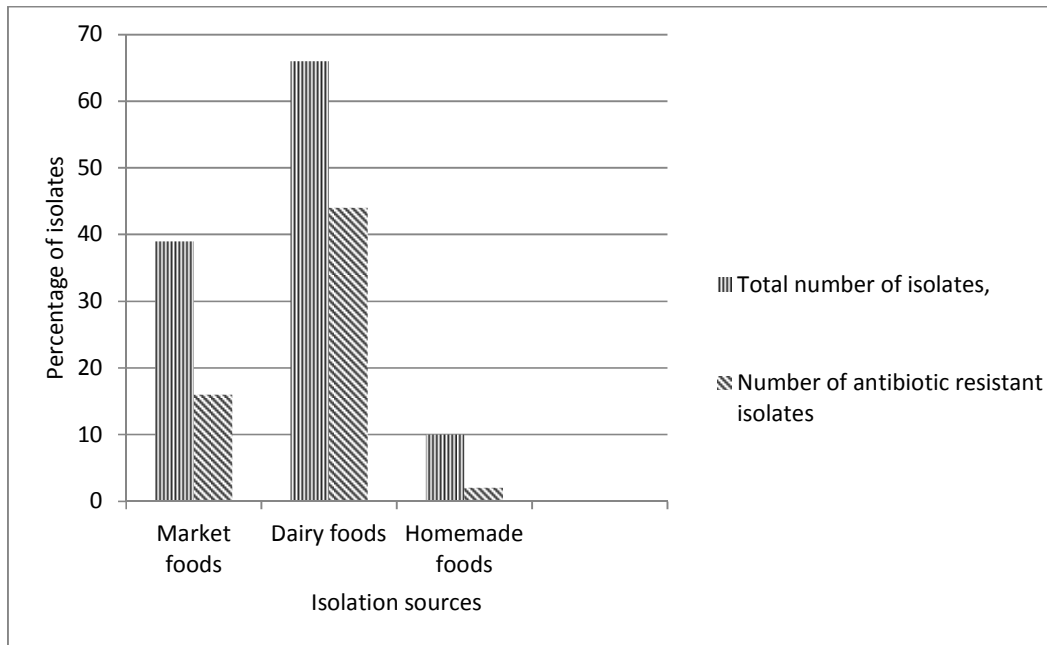

Fig. 1. Percentage of antibiotic resistant strains

Table 5. Percentage of antibiotic resistance strains to total strains according to isolation sources

Isolation source	Resistant strains (Total strains/resistant strains)			
	Lactobacillus spp.	*Lactococcus* spp.	*Enterococcus* spp.	*Streptococcus* spp.
White cheese	1/1	8/1	4/4	9/5
Çökelek	7/6	0/0	0/0	0/0
Kefir	6/6	1/1	0/0	0/0
Sheep cheese	5/4	0/0	1/1	3/2
Lor	14/9	7/0	2/2	2/1
Tulum cheese	16/13	0/0	7/6	3/3
Yoghurt	19/2	0/0	0/0	0/0

chloramphenicol and streptomycin. tet(M) gene reported for the other tetracycline resistant lactococci strains also. In a similar study with raw milk cheese isolates, low frequencies of resistance were detected for tetracyclin (4,3%), gentamycin (17,4%), chloramphenicol (2,2%), erythromycin (2,2%) and lincomycin(2,2%). Antibiotic resistant lactococci might present in cheeses from raw milk of dairy cow which is treated with antibiotics to cure or prevent mastitis. To prevent transfer of antibiotic resistant bacteria from animals into fermented products can be achived by using pasteurize or heat treated raw milk or meat [27].

Among enterococcal isolates antibiotic resistance levels were lincomycin (57,1%), ciprofloxacin (50%), meropenem (35,7%), gentamicin (21,4%), erythromycin (7,1%) and tetracycline (7,1%), and multiresistance phenotype level was 50%. Among these multi-resistant isolates, E. faecalis GLM 183 displayed resistance up to 5 different antibiotics (gentamicin, lincomycin, meropenem, ciprofloxacin, tetracycline) and harboured only tet(M) gene (Table 4). Huys et al. [28] have been reported testracycline resistance in Enteroccus species from European cheeses is correlated with tet(M) gene and also isolates have conjugative transposons which they belong to the Tn916-Tn1545 transposone family. In the study conducted by Frazzon [29] et al. while all Enterococcus isolates from food samples of Brasil were found to be susceptible to vancomycin, high level of tetracycline and erythromycin resistance was observed. The most frequent genotype responsible for tetracycline resistance was tet(M) alone or combination with tet(L). Phenotypic antibiotic resistances of food-borne Enterococci isolated from raw milk, fermented dairy and meat products investigated by many researchers [30,31,32,33]. According to the results of these researchs, while food borne Enterococci are generally resistant to ampicillin and vancomycin, high percentage of multiple antibiotic resistances were determined similar with our results. In another study conducted with Enterococci from milk and cheese samples in Portugal, gentamycin resistance of isolates were investigated. While considered as intrinsically resistant to low concentration to gentamycin, most of Enterococci from dairy samples isolates displayed high level resistance to gentamycin. In our study, PCR amplification of aac(6) aph (2'') gene resulted with positive amplicon for 1 out of 3 gentamycin resistant E. faecalis GLM 132, with MIC value of gentamycin ≥128 µg/mL. Both E. gallinarium strains tested were positive for van(C) gene. Phenotypically, these strains showed moderate resistance to vancomycin (8 µg/mL) and this type of resistance have been reported as spesific to this species [34].

Totally 17 Streptococci strains were isolated in our study and they were susceptible to ampicillin, chloramphenicol, meropenem, ciprofloxacin, teicoplanin and vancomycin. The levels of antibiotic resistance were as follows; tetracycline (47,05%), erythromycin (41,17%), gentamycin (41,17%), lincomycin (23,52%). Of 37 S. macedonicus isolates from Italian raw milk cheeses, all of them were sensitive to clindamycin, co-trimoxazol, erythromycin, gentamicin, penicillin G, tetracycline and vancomycin [35]. While erm(B) and tet(M) genes were detected in all erythromycin and tetracycline resistant S. macedonicus isolates (n=7), tet(L) gene was amplified from three of them. Similar with our results, erm(B) gene was detected in four out of 70 erythromycin resistant S. thermophilus [2]. S. thermophilus is the technologically important species of genus Streptococcus, and has been reported as susceptible to chloramphenicol, tetracycline, erythromycin and ciprofloxacin and resistance to gentamycin at varying degrees [36,37,38]. In our study, all gentamycin resistant S. macedonicus isolates with MIC value of ≥128 µg/mL gave positive results for aac(6) aph(2'') gene. However, one of the S. macedonicus strain, GLM 198, harbouring aac(6) aph (2''), did not show phenotypic resistance. The lack of correlation between resistance phenotype and genotype could be related to defective expression of the resistance gene [29]. S. lutetiensis GLM 150 resistant to gentamycin (MIC value of 64 µg/mL) but aac(6) aph (2'') gene was not detected in this bacterium. The situation is apparent when the phenotypic and genotypic resistance patterns are in agreement, however, a phenotypically resistant bacterium strain may be genotypically "susceptible". This is usually due to the fact that appropriate genes are not included in the test patterns, or there exist unknown resistance genes. Tetracycline, for example, has more than 40 different genes conferring antibiotic resistance discovered and the number of tetracycline resistance genes continues to increase [27]. Seven out of 11 S. macedonicus showed multiresistance to at least two antibiotics. The presence of multiresistant strains, PCR positive results for many resistance genes, indicated that Streptococci is the most risky genera of our study in terms of transfer for antibiotic resistance traits.

5. CONCLUSION

Data from our study indicate that several acquired genes encoding for gentamycin, tetracycline and erythromycin are carried by LAB especially those isolated from cheese samples with dairy origin. According to the BLAST results antibiotic resistance genes detected in LAB have had high homology with those associated with transposons from some pathogenic bacteria. The transmission of gentamycin, tetracycline and erythromycin genes from some Streptococci strains isolated in the study to *E. faecalis* JH2-2 shows that dairy products can be important vehicles for transfer of antibiotic resistant traits.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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