Investigation of Some Antibiotic Resistance Genes of Indigenous *Lactobacilli* Isolated From Traditional Yogurt In Zanjan Province of Iran

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Abstract

Purpose: Given the increasing use of antibiotics on humans and livestock for treatment or as a growth stimulant, antibiotic resistance has become a general concern. The food chain and specially fermented foods could be a source of antibiotic-resistant bacteria and resistance genes. Lactic Acid Bacteria (LAB) and *Lactobacilli* are considered safe to use as starter culture or probiotic strains. Recently, however, antibiotic-resistant genes isolated from LABs showed the necessity of setting international regulations to reduce the risk of antibiotic resistance genes transmission via the food chain. The current study aimed to investigate the antibiotic resistance of *Lactobacilli* isolated from traditional yogurt samples from Zanjan province in Iran.

Methods: *Lactobacilli* characterization and identification were carried out through biochemical and molecular methods. The disk diffusion method was applied to determine phenotype resistance using 13 antibiotic disks resistance genes presence were investigated in the isolates to determine transferability risk, respectively.

Results: Based on biochemical and molecular methods, 24 isolates have been identified as *Lactobacilli* with multiple antibiotic-resistant phenotypes. Vancomycin resistance was a typical phenotype and genotype among isolates. On investigated *Lactobacilli* chromosome, Tetracycline resistance genes Chloramphenicol (cat), beta-lactam, aminoglycosides (aph (3')-III), and aadA resistance genes have been detected. While the examined resistance genes have not been detected on the plasmids, they were all on the bacterial chromosome.

Conclusion: The results showed that the investigated isolates did not carry the resistance genes on their plasmids. It, therefore, would be a good point since they probably do not transfer resistance genes to other bacteria, and they would be proper candidates to do more investigation for introducing new safe starter culture or probiotic strain to food industries.

Introduction

Antibiotics are among those extensive groups of therapeutic agents commonly used in bacterial infection treatments. The term “antibiotic” was first used by Sales Waksman for small molecules produced by microorganisms with antagonistic effects on other species (Ribeiro et al.2019). The antibiotic turning point was in 1928 when Sir Alexander Fleming discovered Penicillin. During World War II, Penicillin was used successfully to treat bacterial infections (Ventola 2015). Fleming was the first to warn of the dangers of Penicillin resistance due to its overuse (Rosenblatt 2009).

Different types of antibiotics can trigger bacterial cell membrane, cell wall, protein, genetic material, and other biologic compounds synthesis (Tortora et al. 2015). Bacteria, however, have flexibility against environmental stresses such as antibiotics. They can resist these molecules through different mechanisms (Munita and Arias 2015) including, altering the antibiotic, permeability changes in the bacterial cell wall, efflux pumps and active transport of the antibiotic, change in antibiotic target structure, antibiotic degradation, and target enzyme overproduction. Resistance to some specific antibiotics in bacteria could be intrinsic, or they could resist antibiotics through mutation or acquiring resistance genes via plasmids or other mobile genetic elements (Peterson and Kaur 2018). Resistant genes were reported for aminoglycosides, β -lactams, Chloramphenicol, macrolides, sulfonamides, Trimethoprim, and Tetracycline (Van Hoek et al.2011).

Antibiotic resistance studies have focused mainly on clinical isolates of bacterial species, but it should be noted that food bacteria might also act as reservoirs of antibiotic resistance genes (Franz et al. 2005). Fermented foods and fermented dairy products may be considered vehicles for transferring antibiotic-resistant bacteria because of their high microbial population (Mathur and Singh 2005). Though *Lactobacilli* have generally known as safe, there are some reports about intrinsic and acquired resistance to antibiotics in some of them like *L.plantarum*, *L. paracasei*, *L.reuteri*, and *L.acidophilus* (D'Aimmo et al.2007;Liu et al;2009Rojo-Bezarez et al.2006), so showing that more investigation on antibiotic resistance in *Lactobacilli* is necessary.

Lactobacilli have intrinsic resistance to aminoglycosides like Gentamycin, Kanamycin, and Streptomycin, while most of them are sensitive to Penicillin and other β -lactams, Chloramphenicol, Tetracycline, and Erythromycin (Abriouel et al.2015). In some cases, acquired resistance to Tetracycline, Erythromycin, and Chloramphenicol has been detected in *Lactobacilli* (Campedelli et al.2018). Therefore, it seems necessary to determine the pattern of antibiotic resistance of *Lactobacilli* and to differentiate between intrinsic and antibiotic resistance. In the present research, antibiotic resistance was investigated in *Lactobacillus* species isolated from traditional yogurt of Zanjan province. Investigation of antibiotic resistance in isolates has been conducted by both phenotypic method and examining some resistance genes.

Material And Methods

Lactobacilli Isolation

Fifty-five (0.5 kg) samples of traditional yogurt were collected from rural areas of Zanjan province in Iran. Samples were immediately taken to the laboratory, stored at 4°C, and cultured in less than 48 hours. For the isolation of *Lactobacillus* from yogurt samples, 1 ml of each sample was used to made serial dilutions with 9 ml of sterile physiological water (NaCl 0.85%). The samples were cultured on MRS agar plates and incubated at 37°C for 24–48 h in anaerobic conditions. All isolates were cultured in MRS broth at 37°C for 24–48 h in anaerobic conditions(Karami et al. 2017), then have stored in MRS broth containing 20% (v/v) glycerol in – 70°C (Ghobadi Dana et al.2011).

Biochemical Identification

Biochemical analysis as Gram stain, catalase, oxidase, indole production, motility, growth at different temperatures (15, 37,45) °C, furthermore, sugars fermentation tests were carried out using sucrose, lactose, maltose, trehalose, galactose, arabinose, mannitol, fructose, as well as ribose (Bin 2018,Ghobadi Dana 2018).

Antibiotic Susceptibility Test

In this research, the disc diffusion method was applied to determine the antibiotic susceptibility of isolated *Lactobacilli*. Isolates have been cultured on nutrient agar for 18-24h, at 37°C under microaerophilic condition transferred to sterile physiologic serum to compare the turbidity of bacterial suspension with Mac Farland standard solution according to Iran National Standard No. 13560 in 625 nm wavelength. The dried surface of the Muller Hinton agar plate was inoculated by bacterial suspension using a sterile swab. Antibiotic disks including, Tetracycline 30µg, Erythromycin 15µg, Kanamycin 30µg, Gentamycin 10µg, Vancomycin 30µg, Penicillin G 10µg, Nalidixic acid 30µg, Rifampin 5µg, and Ciprofloxacin 5µg Chloramphenicol 30µg, Trimethoprim, 5µg Ampicillin 10µg Streptomycin 10µg (Rosco, England, and Fan Azma Iran) were placed on the Muller Hinton agar plates using sterilized forceps. The plates were incubated 16-18-hours at 37°C. For each disk, the apparent zone diameter was measured three times, and for all of them, according to Iran national standard No. 13560, standard deviation was calculated.

Molecular Characterization

By ready-to-use Yakhteh Saba Arena Company in Iran, as constructor instruction, Chromosomal DNA was extracted. Molecular characterization of *Lactobacilli* carried out by specific 16S rDNA sequence as follows: R16-1: (5'-CTTGTACACACCGCCGTC - 3', LbLMA1-rev: 5'-CTCAAACTAAACAAAGTTTC-3'). PCR reaction was carried out in Biorad 1000 thermal cycler using Sina clone master mix 25µl, template DNA 1µl, forward primer 1µl, reverse primer 1µl, and sterile distilled water to a final concentration of 50µl (Saavedra et al.2004). PCR reaction cycles were carried out with the following condition, denaturation at 95°C for 5 minutes, followed by 30 cycles for 40 seconds at 95°C. Annealing was done at 57.6°C for 40 seconds. Elongation was carried out at 72°C for 40 seconds, followed by final elongation at 72°C for 5 minutes (Hudzicki2009,Ghobadi Dana 2018). Then, gel electrophoresis was carried out with agarose 1% in TAE buffer (40 mMTris-acetate, 1mM EDTA, pH 8.2) and stained with ethidium bromide visualized by a gel documentation device.

Antibiotic Resistance Genes Detection

By ready-to-use Yakhteh Saba Arena Company in Iran, chromosomal DNA was extracted. To extract plasmid DNA, PishgamanEnteghal Gene 50 reaction kit was used based on alkaline lysis using RNase to minimize RNA and genomic DNA pollution. Plasmid extraction steps were following according to kit instructions. According to Table 3, the polymerase chain reaction was carried out to detect antibiotic resistance genes in chromosomal DNA and plasmid DNA separately, using specific primers synthesized by Dena Zist Asia, Iran, and Sina Clone Company. The PCR reaction was carried out in Bio-Rad 1000 thermal cycler with the respective conditions, described for each resistance gene separately. Agarose gel electrophoresis was carried out for each resistance gene, with agarose 1% in TAE buffer (40 mMTris-acetate, 1 mM EDTA, pH 8.2) and stained with ethidium bromide then visualized by gel documentation device. Primers and amplicons sizes for each are listed in Table1.

Table 1
Primers and amplicon sizes and annealing temperature for each primer are shown.

Antibiotic	Gene Name	Primers	Amplicon size (bp)	Annealing Temperature	reference
Tetracycline	tet (M)	GTGGACAAAGGTACAACGAG CGG TAA AGT TCG TCA CAC AC	406	55	(Aimov et al. 2007)
	tet (W)	GAGAGCCTGCTATATGCCAGC GGG CGT ATC CAC AAT GTT AAC	168	55	(Aimov et al. 2007)
Erythromycin	erm (B)	TGGTATTCCAAATGCGTAATG CTG TGG TAT GGC GGG TAA GT	745	61.8	(Dec et al. 2017)
Chloramphenicol	cat	TAAGGTTATTGGGATAAGTTA GCATGRTAACCATCACAWAC	340	54	(Devirgiliis et al. 2008;Guo et al.2017)
βlactams	bla Z	ACTTCAACACCTGCTGCTTTC TAG GTT CAG ATT GGC CCT TAG	240	60	(Dušková et al. 2017;Guo et al.2017)
Aminoglycosides	aac(6") - aph (2")	CCAAGAGCAATAAGGGCATACC ACCCTCAAAACTGTTGTTGC	675	58	(Belletti et al. 2008;D'Aimmo et al. 2007; Rojo-Bezares et al. 2006))
	aph (3")-III	GCCTTTCGCCACCTCACCG GCCGATGTGGATTGCGAAAA	292	55	;Kobayashi et al. 2001) (Dušková et al2017;Qouba et al.2008);
	aad A	CCTCGTGAATTCATGTTCTGGC ATCCTTCGGCGCGATTTTG	282	56	
Vancomycin	van A	GGGAAAACGACAATTGC GTACAATGCGGCGTTA	732	59	(Tenorioet al.2001)

Tetracycline Resistance Genes PCR Amplification

Two common genes studied, including tet (M) and tet (W), were used to evaluate Tetracycline resistance. The polymerase chain reaction was done to detect these genes using specific primers for tet (M): F: 5'GTG GAC AAA GGT ACA ACG AG 3', R: 5'CGG TAA AGT TCG TCA CAC AC 3', and tet (W): F: 5' GAG AGC CTG CTA TAT GCC AGC 3' R: 5'GGG CGT ATC CAC AAT GTT AAC 3'. PCR carried out using Sinaclon master mix 25µL, forward primer 1µL, reverse primer 1µL, 1µL of template DNA, and DNA/ RNA free distilled water to reach the final volume of 50µL. The polymerase chain reaction was carried out as following amplification program: Initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45s, annealing at 55°C for 1 min, (Aminov et al. 2007) and extension at 72°C for 1 min. A final extension was performed at 72°C for 5 minutes (Hedayatianfard et al. 2014). All samples were tested twice. Positive control was the tet (M), tet (w) positive *Lactobacillus* (Hedayatianfard et al.2014).Using agarose 1% in TAE buffer (40 mMTris-acetate, 1 mM EDTA, pH 8.2), agarose gel electrophoresis was carried out, stained with ethidium bromide, and then visualized by a gel documentation device.

Erythromycin Resistance Gene (erm (B)) PCR Amplification

Polymerase chain reaction carried out using specific primer for erm (B) gene: F: 5'TGG TAT TCC AAA TGC GTA ATG 3'and R: 5'CTG TGG TAT GGC GGG TAA GT 3'. Using 12.5µL Sina Clone company PCR master mix, forward primer 1µL, reverse primer 1µL, 1µL of template DNA, the polymerase chain reaction were done and DNA/ RNA free distilled water to reach the final volume of 25µL (Aminov et al.2004). At the following conditions, DNA amplification for erm (B) gene was performed: Initial denaturation at 93°C for 3 minutes, followed by 30 cycles of denaturation at 93°C for 1 minute, annealing at 61.8°C for 1 minute, (Dec et al.2017) extension at 65°C for 1 minute, and final extension for 3 minutes after final cycle (Aminov et al.2004). All samples were tested twice. Positive control was the erm (B) positive *Lactobacillus*. Agarose 1% in TAE buffer (40 mMTris-acetate, 1 mM EDTA, pH 8.2), eventually, agarose gel electrophoresis was done, followed by staining with ethidium bromide, then visualized by a gel documentation device.

Chloramphenicol Resistance Gene (cat) PCR Amplification

Detection of cat gene was done using PCR reaction with specific primer F: 5'TAA GGT TAT TGG GAT AAG TTA 3'and R: 5'GCA TGR TAA CCA TCA CAW AC 3'the polymerase chain reaction was conducted as following using 12.5µL, forward primer 1µL, reverse primer 1µL, 1µL of template DNA, and DNA/ RNA free distilled water to reach the final volume of 25µL (Anisimova and Yarullina 2018). At the following conditions, DNA amplification for cat gene was performed: initial denaturation at 94°C for 5 min, 30 cycles denaturation at 94°C for 45 s, annealing at 54°C for 45 s, and extension at 72°C for 75 s and a final extension step at 72°C for 8 min (Guo et al.2017).All samples were tested twice. Positive control was the cat gene-positive *Lactobacillus*. Using 1% agarose in TAE buffer (40 mMTris-acetate, 1 mM EDTA, and pH 8.2), agarose gel electrophoresis was conducted, and staining was done with ethidium bromide and then visualized by a gel documentation device.

β-Lactam Resistance Gene Detection (bla Z)

The PCR reaction, in this research, was carried out to determine the resistance to Penicillin and Ampicillin among isolates, PCR reaction using specific primer for (bla Z) gene: F: 5'ACT TCA ACA CCT GCT GCT TTC 3'and R: 5'TAG GTT CAG ATT GGC CCT TAG 3'. The reaction was conducted using Sina clone master mix 25µL, forward primer 1µL, reverse primer 1µL, 1µL of template DNA, and DNA/ RNA free distilled water to reach the final volume of 50µ.

Cycling conditions for bla Z gene amplification were as follows: denaturation at 95°C for 5min, followed by 35 cycles of 94°C for 30 s, annealing temperature at 60°C for 30 s, extension at 72°C for 60 s and final extension of 72°C for 10 min (Guo et al.2017). All samples were tested twice. Positive control was the bla Z gene-positive *Lactobacillus*. Using 1% agarose in TAE buffer (40 mMTris-acetate, 1 mM EDTA, pH 8.2) to detect the β-lactam resistance gene, agarose gel electrophoresis was done and stained with ethidium bromide, and then visualized by a gel documentation device.

Aminoglycosides Resistance Genes Detection

In the present study, for Gentamycin, aac (6") – aph (2") (Abraham and Sistla, 2017), Kanamycin aph (3")-III, and Streptomycin aad A (Goudarziet al.2020), Aminoglycoside resistance genes were investigated. Specific primers for each gene were used including, aac (6") aph(2"): F:5'CCAAGAGCAATAAGGGCATACC 3', R: 5' ACCCTCAAAAAGTGTGTTGC 3' aph(3")III: F: 5'GCCTTTCGCCACCTCACCG3', R:5'GCCGATGTGGATT CGAAAA 3', and aad A F: 5'CCTCGTGTAATTCATGTTCTGGC 3', R: 5'ATCCTTCGGCGCATTTTG 3'. Using ready-to-use master mix 25µL, forward primer 1µL, reverse primer 1µL, 1µL of template DNA, and add DNA/ RNA free distilled water to reach the final volume up to 50µL.

Aminoglycoside resistance genes including aac (6") – aph (2"), aph (3")-III, and aad A were detected as the following condition: initial denaturation at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 1 minute, annealing for 45 seconds at 58°C for aac (6") - aph(2"),55°C for aph(3")III (Kobayashi et al. 2001)and 56°C for aadA gene (Qouba et al.2008) and extension at 72°C for 30 seconds (Vakulenko and Mobashery2003). Using 1% agarose in TAE buffer (40 mMTris-acetate, 1 mM EDTA, and pH 8.2), agarose gel electrophoresis was carried out, and staining was done with ethidium bromide and then visualized by a gel documentation device.

Vancomycin Resistance Gene (vanA) Detection

The PCR reaction was conducted with a specific primer for van(A) gene to investigate Vancomycin resistance: F: 5'GGGAAAACGACAATTGC 3' and R: 5' GTACAATGCGGCGTTA 3', using ready to use master mix 25µL, forward primer 1µL, reverse primer 1µL, 1µL of template DNA, and DNA /RNA free distilled water to reach the final volume of 50µL.

Vancomycin resistance gene PCR amplification program was as follows: denaturation step at 94°C for 5 minutes, followed by 30 cycles of 45 seconds at 94°C, 45 seconds at 59°C, and 45 seconds at 72°C. A final extension step was performed at 72°C for 5 minutes (Tenorio et al.2001). Agarose gel electrophoresis was then carried out with 1% agarose in TAE buffer, including (40 mMTris-acetate, 1 mM EDTA, pH 8.2), and then stained with ethidium bromide, finally visualized using a gel documentation device.

Findings

Lactobacilli Isolation

Lactobacillus colonies were white, circular, and they were gram-positive as well as rod-shaped, in microscopic evaluation.

Biochemical Identification

The Biochemical tests, including catalase, oxidase, indole production, and motility growth at different temperatures (15–37–45), furthermore, sugar fermentation tests were carried out. Consequently, *Lactobacilli* isolates were catalase and oxidase negative, and did not produce indole, non-motile, moreover, could grow on 15, 37, and 45°C. The sugar fermentation tests were carried out using sucrose, glucose, lactose, maltose, trehalose, galactose, arabinose, mannitol, fructose, and ribose. All of the isolates could ferment glucose and lactose; 75% of isolates were fermented galactose, 62.5% fermented fructose, 45% could ferment sucrose, 33% arabinose, and 29% of our isolates fermented maltose, and any of our isolates could ferment ribose, 8% mannitol, and trehalose. The results of the sugar fermentation test for the isolates are shown in Table 2.

Table 2
Shows the Results of Sugar Fermentation Tests for Each *Lactobacilli*

Isolates	Lactose	Glucose	Fructose	Galactose	Ribose	Trehalose	Arabinose	Maltose	Mannitol	Sucrose
No. 1	+	+	+	+	-	-	-	-	-	+
No. 2	+	+	-	+	-	-	-	+	-	+
No. 3	+	+	-	+	-	-	+	+	+	+
No. 4	+	+	+	+	-	+	-	-	+	-
No. 5	+	+	-	-	-	-	+	-	-	+
No. 6	+	+	-	+	-	-	-	-	-	+
No. 7	+	+	+	+	-	+	+	-	-	+
No. 8	+	+	+	+	-	-	-	-	-	-
No. 9	+	+	+	+	-	-	-	-	-	+
No. 10	+	+	+	+	-	-	+	+	-	-
No. 11	+	+	+	+	-	-	-	-	-	-
No. 12	+	+	-	-	-	-	-	-	-	-
No. 13	+	+	-	-	-	-	+	+	-	-
No. 14	+	+	+	+	-	-	+	+	-	+
No. 15	+	+	-	-	-	-	-	-	-	-
No. 16	+	+	+	+	-	-	-	+	-	-
No. 17	+	+	-	+	-	-	-	-	-	-
No. 18	+	+	+	-	-	-	-	-	-	+
No. 19	+	+	+	+	-	-	+	-	-	-
No. 20	+	+	+	+	-	-	-	+	-	-
No. 21	+	+	+	+	-	-	+	-	-	+
No. 22	+	+	-	+	-	-	-	-	-	+
No. 23	+	+	+	+	-	-	-	-	-	-
No. 24	+	+	+	-	-	-	-	-	-	-

Antibiotic Susceptibility Test

Zone diameter charts are listed in Table 3. To determine antibiotic sensitivity and resistance according to CLSI 2018 (Performance Standards for Antimicrobial Susceptibility Testing 2018).

Table 3
Shows Disk Zone Diameter Chart for the Determination of
Antibiotic Sensitivity and Resistance According to CLSI 2018

Antibiotic Name	Resistant	Intermediate	Sensitive
Tetracycline 30µg	≤ 14	15–18	≥ 18
Erythromycin 15 µg	≤ 13	14–22	≥ 23
Ampicillin 10µg	≤ 13	13–14	≥ 15
Penicillin G 10µg	≤ 14	—	≥ 15
Vancomycin 30µg	≤ 14	15–16	≥ 17
Kanamycin 30µg	≤ 13	14–17	≥ 18
Gentamycin 10µg	≤ 12	13–14	≥ 15
Streptomycin 10µg	≤ 11	13–16	≥ 17
Ciprofloxacin 5µg	≤ 15	16–20	≥ 21
Trimethoprim 5µg	≤ 10	11–15	≥ 16
Chloramphenicol 30µg	≤ 12	13–17	≥ 18
Rifampin 5µg	≤ 16	17–20	≥ 21
Nalidixic Acid 30µg	≤ 13	14–18	≥ 19

Antibiotic susceptibility test was performed by the disk diffusion method and the results are mentioned in Table 4.

Table 4
Shows Clear Zones Diameter Average after Measuring Three Times for Each Iso

Antibiotic / Inhibition Zone Diameter	Vancomycin	Chloramphenicol/	Penicillin/ Inhibition zone diameter	Ampicillin/ Inhibition zone diameter	Erythromycin/ Inhibition Zone Diameter	Tetracycline/ Inhibition Zone Diameter	Gentamycin/ Inhibition Zone Diameter	Kanamycin/ Inhibition Zone Diameter	Trimetoprim/ Inhibition Zone Diameter
No. 1	16 ± 0.11	18 ± 0.20	0.02 ± 10	13 ± 0.01	18 ± 0.009	19 ± 0.01	12 ± 0.09	12 ± 0.02	17 ± 0.09
No. 2	17 ± 0.09	19 ± 0.17	16 ± 0.09	17 ± 0.26	12 ± 1.116	19 ± 0.52	22 ± 0.09	17 ± 0.17	16 ± 0.26
No. 3	18 ± 0.42	18 ± 0.47	13 ± 0.33	15 ± 0.10	12 ± 0.21	11 ± 0.17	21 ± 0.44	12 ± 0.25	10 ± 0.29
No. 4	12 ± 0.13	18 ± 0.35	12 ± 0.17	14 ± 0.02	12 ± 0.25	14 ± 0.20	22 ± 0.08	14 ± 0.12	10 ± 0.25
No. 5	10 ± 0.60	20 ± 0.06	15 ± 0.02	18 ± 0.004	19 ± 0.6	19 ± 0.066	11 ± 0.17	18 ± 1.10	18 ± 0.06
No. 6	15 ± 0.08	18 ± 0.41	14 ± 0.091	12	14 ± 1.03	15 ± 0.08	23 ± 0.5	14 ± 0.002	14 ± 0.9
No. 7	16 ± 0.012	18 ± 0.06	20 ± 0.01	11 ± 0.12	19 ± 0.42	19 ± 0.71	22 ± 0.6	18	18 ± 1.3
No. 8	15 ± 0.50	12	14 ± 0.05	11 ± 0.06	18 ± 0.51	13 ± 0.23	12 ± 0.11	18 ± 0.40	14 ± 0.67
No. 9	13 ± 0.32	18 ± 0.03	17 ± 0.02	14 ± 1.00	15 ± 0.02	16 ± 0.10	23 ± 0.40	17 ± 0.05	15
No. 10	10 ± 0.8	19 ± 0.10	15	17 ± 0.50	14 ± 0.30	18 ± 0.01	22	22 ± 0.07	18 ± 0.06
No. 11	12 ± 0.03	18 ± 0.51	17 ± 0.10	17 ± 0.40	16 ± 0.04	12 ± 0.05	22 ± 0.10	15 ± 0.14	18 ± 0.01
No. 12	18	11 ± 0.02	20 ± 0.15	14 ± 0.09	18 ± 0.30	19 ± 0.20	21 ± 0.80	10 ± 0.70	22 ± 0.76
No. 13	11 ± 0.20	19 ± 0.50	15 ± 0.11	16 ± 0.40	19 ± 0.55	23 ± 1.05	11 ± 0.05	12 ± 0.17	17 ± 0.01
No. 14	10 ± 0.08	12 ± 0.30	20 ± 0.50	13 ± 0.50	11 ± 0.45	21 ± 0.03	22 ± 0.70	24 ± 1.05	10 ± 0.80
No. 15	12 ± 0.10	14 ± 0.47	26 ± 1.02	18 ± 0.75	18 ± 0.67	20 ± 0.08	22 ± 0.85	15 ± 0.07	19 ± 0.75
No. 16	12 ± 0.50	18 ± 0.50	23 ± 0.80	15 ± 0.04	12 ± 0.40	19 ± 0.05	21 ± 0.01	13 ± 0.40	17 ± 0.30
No. 17	10 ± 0.06	19 ± 1.00	21 ± 0.47	12 ± 0.06	19 ± 0.25	19 ± 0.40	18 ± 0.06	19 ± 0.85	10 ± 0.05
No. 18	19 ± 1.11	18 ± 0.90	25 ± 0.56	14 ± 0.70	16 ± 0.18	15 ± 0.05	18 ± 0.03	16 ± 0.70	20 ± 0.80
No. 19	15 ± 0.05	18 ± 0.30	18 ± 0.50	11 ± 0.30	18 ± 0.75	19 ± 0.61	21 ± 0.75	25 ± 1.02	15 ± 0.75
No. 20	18 ± 0.20	18 ± 0.45	22 ± 0.32	18 ± 0.60	22 ± 0.15	12 ± 0.50	13 ± 0.45	18 ± 0.01	9 ± 0.08
No. 21	19 ± 1.50	20 ± 0.50	16 ± 0.75	20 ± 0.90	21 ± 0.50	16 ± 0.45	16 ± 0.02	17 ± 0.90	23 ± 1.06
No. 22	17 ± 0.05	10 ± 0.17	14 ± 0.45	12 ± 0.71	19 ± 0.04	22 ± 0.06	21 ± 0.65	12 ± 0.06	18 ± 0.03
No. 23	17 ± 0.02	12 ± 0.06	11 ± 0.80	12 ± 0.50	18 ± 0.30	12 ± 0.40	14 ± 0.03	10 ± 0.05	19 ± 0.002
No. 24	19 ± 0.45	18	21 ± 0.04	18 ± 0.60	19 ± 0.65	17 ± 0.01	20 ± 0.29	14 ± 0.03	17 ± 0.01

Clear zones diameter was analyzed using CLSI 2018 for each antibiotic on the Muller Hinton agar (Guo et al.2017).Vancomycin resistance was the typical phenotype among the investigated isolates with 45.8%, and other antibiotic-resistant phenotypes were as follows: Ampicillin 37.5%, Penicillin 33.3% Kanamycin 29.16%, Tetracycline, and Gentamycin, both 25%, Ciprofloxacin, Erythromycin, Chloramphenicol, Trimethoprim are 20.8%, Nalidixic Acid 12.5%, and Streptomycin 8.33%. There was no resistance to Rifampin among isolates. Some isolates, in the present research, have shown resistance to more than one antibiotic.

The disk diffusion results for Lactobacillus isolated from traditional yogurt using Ciprofloxacin, Penicillin, Gentamycin, Tetracycline, Vancomycin, and Erythromycin antibiotic disks on the Muller Hinton agar are shown in Fig. 1.

There were two isolates with resistance to seven antibiotics, including isolates No. 3 with resistance to (Penicillin, Erythromycin, Tetracycline, Gentamycin, Kanamycin, Trimethoprim, Ciprofloxacin), and No. 8 (Chloramphenicol, Ampicillin, Tetracycline, Gentamycin, Nalidixic acid, and Ciprofloxacin, Penicillin). Furthermore, six isolates were resistant to five antibiotics (isolates Nos. 1, 4, 14, 17, 20, and 23). The isolate No. 22 was resistant to four antibiotics, including (Chloramphenicol, Penicillin, Ampicillin, and Kanamycin). There were four with resistance to three antibiotics (2, 6, 13, and 16). Moreover, three isolates were resistant to two antibiotics (9, 11, and 12). Four isolates were resistant to one antibiotic (7, 10, 15, and 19). Given the investigated antibiotics, three isolates were not resistant (18, 21, and 24).

DNA Extraction

D value for DNA extracts determined using a spectrophotometer at 260 nm and 280 nm. The ratio of (260/280) indicated the concentration of DNA in the samples, which was about 1.9 for investigated samples.

Plasmid DNA Extraction

The ratio of absorbance at 260 nm to 280 nm was calculated. The ratio for plasmid extracts was 1.8 in this research, which shows the acceptable purity of plasmid DNA.

Molecular Characterization

Identification of *Lactobacilli* carried out by polymerase chain reaction using 16S rDNA sequence. Finally, according to the 16S rDNA sequence, 24 isolates were identified, as shown in Fig. 2.

Antibiotic Resistance Genes Detection

Tetracycline Resistance Genes Detection

Detection of tet (W)

Among the investigated *Lactobacilli*, only one (isolate No. 22) had the tet (W) gene on its chromosome. Electrophoresis results for chromosome DNA extracts are shown in Fig. 3. Amplicon sizes for tet (W) were 168 bp, and it is one of the main Tetracycline resistance determinants. Plasmid extracts were also examined to identify this gene, but none was found.

Detection of tet (M)

Tetracycline resistance gene, including tet (M) was investigated on chromosomal and plasmid DNA of isolates. Amplicon size for tet (M), was about 406 bp, tet (M) was detected on chromosomal DNA of isolates Nos. 3 and 4. Agarose gel electrophoresis results for tet (M) are shown in Figs. 15–18. No one of the isolates had the tet (M) on their plasmid.

Erythromycin Resistance Gene Detection

Erythromycin resistance gene (erm (B)) was investigated in isolates. None of the isolates had an Erythromycin resistance gene on their chromosomal DNA. In bacterial plasmids, the erm (B) gene was detected and was not found in them. Figures 26 – 23 show the agarose gel electrophoresis results for the erm (B) gene in extracts of the bacterial chromosome isolated from *Lactobacillus*, which was not found in the isolated chromosomes

Chloramphenicol Resistance Gene Detection

The Chloramphenicol resistance gene (cat) was detected on isolates Nos. 8, 12, 14, while five *Lactobacilli* (including 8, 12, 14, 23, and 24) were phenotypically resistant to this antibiotic; moreover, the cat gene was not detected in Nos. 13 and 17 of isolated *Lactobacilli*. Electrophoresis on agarose gel results for the chloramphenicol (cat) resistance gene is shown to be 340 bp amplicon size. The detection of cat genes on bacterial plasmid extracts was done and not found in any investigated isolates.

β -lactam Resistance Genes detection

The bla Z gene, related to penicillin resistance, was identified in six (Nos. 3,4,5,9, 22, and 23) *lactobacilli* isolates. The amplicon size of PCR products for the bla Z gene was about 238 bp, as shown in figures 38-41. The bla Z gene presence on the bacterial plasmid was investigated, and none of the isolates contained this gene on their plasmid. Figures 42-44 show the results of agarose gel electrophoresis. Ampicillin resistance also could be the result of the bla Z gene (Brinas et al. 2002). Isolates No. 1, 6, 8,14,17,19, 22, and 23 were Ampicillin resistant phenotypically.

Aminoglycoside Resistance Genes Detection

aac(6") - aph(2") Gene Detection

The presence of aac (6") - aph (2") on bacterial chromosome and plasmid has been investigated for resistance to Gentamicin and Kanamycin (Goudarziet al.2020), which was not observed in the isolates.

Detection of aph(3")-III

aph (3") -III belongs to kanamycin, and other aminoglycosides (Vakulenko and Mobashery 2003) was detected in one of the isolated chromosome DNAs (No. 20). On isolates plasmid extracts, this resistance gene was not detected.

aad (A) Gene Detection

This aminoglycoside resistance gene was found in six isolated *Lactobacilli* (including Nos. 3, 4, 5, 6, 13, and 16). Aminoglycoside resistance genes were not found in the isolates bacterial plasmid. Aminoglycoside resistance genes were not found in the isolates bacterial plasmid. For six chromosomal extracts, the results of agarose gel electrophoresis for the aadA gene are shown in figure 4.

Vancomycin Resistance Gene Detection

Vancomycin resistance gene van (A) was the most prevalent among studied isolates, and seven of the isolates (Nos. 4, 5, 8, 9, 10, 11, 12, 13) had this gene on their chromosome, agarose gel, while in phenotypic analysis (Nos. 4, 5, 9, 10, 11, 13, 14, 15, 16, 17, 18) have shown resistance to Vancomycin.

Using PCR, the van (A) gene on the bacterial plasmid was detected and was not found in any isolates. The agarose gel electrophoresis results to detect the van (A) gene in seven isolated plasmids are shown in figure 5.

Discussion And Conclusion

Traditional fermented dairy products are a good source for the isolation of *Lactic acid bacteria*, especially *Lactobacillus* (Soleimanifard et al. 2015). Since fermented dairy products could act as a reservoir of antibiotic resistance genes, they can transmit resistant genes to commensal or pathogenic bacteria (Erginkaya et al. 2018). Though *Lactobacilli* are commonly not recognized as pathogens, the main concern concerns transferring resistance genes to commensal or pathogenic bacteria. Antibiotic resistance genes expression and transferring resistance determinants to the regular human or pathogenic bacteria are essential components of the food enterprise's safety evaluation and should be considered (EUCAST 2019). The prevalence of antibiotic resistance in *Lactobacillus* isolated from dairy products has been studied in different regions. Using the disk diffusion method, Wang et al. (2018) had investigated antibiotic resistance of LAB isolated from commercial yogurt and cheese EUCAST (2019), Guo et al.(2017) isolated thirty-three strains of *Lactobacillus* from fermented milk collected from different areas of China. Then antibiotic resistance genes were examined in the isolated *Lactobacilli*. Numerous studies on traditional dairy products have shown that these products have unique microflora that depends on the process conditions as well as their ecological production location (Ghobadi Dana et al.2010).

Erginkaya et al. (2018) have investigated the antibiotic resistance of *Lactic acid bacteria* (LAB) isolated from fermented Turkish dairy products (Wang et al.2018). The number of *Lactobacilli* isolated from traditional yogurt was 24, and the diffusion disk method was used to determine antibiotic resistance, using the 13 antibiotic disks as stated. In 45% of isolates, Vancomycin resistance was observed and was the most common phenotypic resistance. The results are consistent with Hagshenas et al. (2015), Erginkaya et al. (2018),Wang et al. (2019). Traditional yogurt samples are collected from rural areas, where farmers do not use antibiotics as growth stimulants and make yogurt using a traditional early culture. Hagshenas et al. (2015) had isolated LAB from traditional dairy products but did not study the presence of antibiotic resistance genes (Hagshenas et al.2015).

Lactobacillus species are usually sensitive to beta-lactam antibiotics (Kaczorek et al.2017), but recent studies have shown resistance to Penicillin G and Ampicillin. Penicillin and Ampicillin resistant isolates were observed in this research. The results of the study are by the following Belletti et al.(2009), Danielsen and Wind (2003), Čanžek et al. (2007), Flórez et al. (2005)Temmerman et al. (2003). In this research, resistance to penicillin in eight isolates (1, 3, 4, 6, 8, 9, 22, and 23) and resistance to ampicillin in (37.5%) 9 isolates (Nos. 1, 6, 7, 8, 14, 17, 19, 22, and 23) were observed. Four isolates, including Nos. 1, 17, 22, and 23, have shown resistance to penicillin and ampicillin together. The bla Z gene can typically be responsible for penicillin, and in some cases, it is resistant to Ampicillin (Patterson et al.1989) and found in isolates Nos.4, 3, 1, 22, 9, and 23. Phenotype and genotype correspondence was 75% in penicillin-resistant isolates and 66.7% in Ampicillin.

Lactobacilli, generally, are resistant to low concentrations of protein synthesis inhibitors; in most cases, this is an inherent feature, although some *Lactobacilli* may be resistant to some of these antibiotics (Campedelli et al.2018).

Resistance to Tetracycline, in general, has been reported from *Lactobacillus* isolated from different foods. Bellelti et al.(2009) on *Lactobacillus* isolates of Italian cheese has shown that 5% of the isolates were resistant to Tetracycline, and in the study by Erginkaya et al. (2018) and Bellelti et al. (2009), 4% of the isolates were resistant to Tetracycline. In this research, Tetracycline resistance was observed in 25% of the isolates (Nos. 3, 4, 8, 11, and 20), which is in line with the opinion of Başbülbül et al.(2015), who showed 30% of the isolates were Tetracycline resistant.

Since Tetracycline resistance is a common feature in *Lactobacilli* and tet (M), in this study, the presence of tet (M) and tet (W) for Tetracycline was investigated. In this research, two isolates (No. 3 and 4) contained tet (M), and one of them (No. 8) had tet (W) on its chromosome. The prevalence of tet genes among *Lactobacilli* isolated from traditional yogurt in Zanjan province in Iran is low, consistent with the results of similar studies such as Ledina and others (Ledina et al.2018). Tetracycline resistance could be transmissible in *Lactobacillus* species, and some studies such as Devirgiliis et al. (2008), Dec et al.(2017) have identified conjugative transposon Tn916 to carrying the tet (M) gene, which can be transmitted from *L. paracasei* isolates to the opportunistic pathogen *Enterococcus faecalis*.tet (M) and tet (W), in this research, were located on bacterial chromosomes, which is in contrast with Devirgiliis et al.(2008) results from phenotype and genotype compatibility in Tetracycline resistance was 60%, and 3 of 5 phenotype-resistant isolates contained Tetracycline resistance determinants, indicating that other resistance determinants could play a role (Devirgiliis et al.2008).

Most *Lactobacillus* species, generally, are susceptible to Erythromycin (Ammor et al.2007) and erm (B), which is encoding an rRNA methylase acting on the 23S ribosomal subunit representing the most widespread resistance genes among other resistance determinants for these two antibiotics (Weisblum et al.2014). Hence, the resistance and erm (B) for Erythromycin resistance in this research were investigated. Obtained results aline with research on *Lactobacillus* strains isolated from Serbian raw milk cheese and other studies such as Ammor et al. (2008b) and Comunian et al. (2010).

The Erythromycin-resistant phenotype was observed in 20% of isolates (2, 3, 4, 14, 16), and the results are consistent with the results of studies by Erginkaya et al. (2018) and Guo et al. (2017). Erythromycin is an antibiotic from the macrolides class, which has been used widely as growth stimulant. The erm (B) gene, as mentioned above, was tested in isolates but ultimately not detected in isolates. It shows that possibly other resistance genes or determinants are responsible for Erythromycin resistance in isolates. The results are in contradiction with the results of Anisimova and Yarullina (2018) because erm (B) was located on the bacterial chromosome in *L. fermentum* and has often been reported in *Lactobacilli* isolates from various sources (Anisimova and Yarullina2018; EUCAST 2019; Wang et al.2018).

Aminoglycoside resistance is a common feature in *Lactobacillus* species. It is usually inherent, resulting from a lack of an electron transfer system for antibiotic uptake, alteration of cell permeability, and the use of enzymatic modification (Charteris et al. 2001; Jaimee and Halami2016).In this research, the

presence of genes related to enzyme modification could be transferable (Turnidge and Bell 2005) in isolates was investigated. The results showed resistance to Kanamycin, Gentamycin, and Streptomycin.

Gentamicin resistance was observed in 25% of isolates (1, 3, 8, 20, and 23). The results are in line with Erginkaya et al. (2018) research, in which 28% of the isolates showed phenotypic resistance to Gentamicin. Kanamycin resistance was shown in seven isolates, including (Nos. 1, 3, 12, 13, 16, 22, and 23), equal to 29.16%, which the results are in line with Campdelli et al. (2018). Streptomycin resistance was not a prevalent phenotype in isolates, and it was observed in 2 isolates (Nos. 6, 13) equal to 8.33% of isolates, which the results were in line with Campdelli et al. (2018); this means the prevalence of Streptomycin resistance is low. The results contradict the findings of Wang et al. (2018), who found that the prevalence of streptomycin-resistant isolates was about 44% of the total and was relatively high. Gentamicin, Kanamycin, and Streptomycin modifying genes are frequently found in LAB isolated from farms. Kanamycin and Streptomycin resistance genes have often been reported in different isolates of *Lactobacillus* (Haghshenas et al. 2015; Dec et al. 2017). In this research, the presence of resistance genes to the mentioned aminoglycosides was investigated. *aph* (3'')-III, which is related to Kanamycin resistance, in one of the isolates (No. 20), *aadA*, which is responsible for Streptomycin resistance found in 6 isolates (3, 4, 5, 6, 13 and 16), *aac* (6')-*aph* (2'') which is related to aminoglycosides, except for Streptomycin resistance and is commonly responsible for Gentamycin resistance (Danielsen and Wind 2003) was not detected in isolates. In the phenotypic analysis, seven of the isolates showed resistance to Kanamycin. At the same time, one of them (No. 20) carried out *aph* (3'') III, which phenotype and genotype accordance is 14%, and it shows other mentioned mechanisms are responsible for resistance, or other resistance genes, which were not investigated in this research. According to Zhou et al. (2005), five of 17 Kanamycin-resistant *Lactobacilli* phenotypes contained *aph* (3'') III; resistant phenotypes, in the present research, are more than genotypes and were the same to present study's results. The streptomycin resistance gene, in this research, was detected in six isolates while two of the isolates were phenotypically resistant, and six isolates were resistant in phenotypical analysis. The Gentamycin resistance gene was not detected among isolates. Therefore, genotype and phenotype were not found in *aac* (6') - *aph* (2''), and it seems other mechanisms such as decreased aminoglycoside uptake appear to reduce cell permeability (Haghshenas et al. 2015). Two of the isolates (6 and 13) were phenotypically Streptomycin resistant, while *aad* (A) was found in six isolates in which the four other isolates (3, 4, 5, and 16) were intermediate phenotype, according to inhibition zone diameter.

In this study, (20.8%) five isolates (8, 12, 14, 22, and 23) have shown Chloramphenicol resistance, which the results were in line with Erginkaya et al. (2018), Dec et al. (2017) and Campdelli et al. (2018). For detecting Chloramphenicol resistance, *cat* gene primer was used, and three isolates (14, 12, and 8) contained *cat* on their chromosome. In Sakumarini et al. (2014), while phenotypic resistance was observed, the *cat* gene was not detected in isolates. The results of this study have conformity with Sakumarini et al. (2014), as some resistant phenotypes were not matched with genotypes. The phenotype and genotype accordance was 60% in this research it shows that probably other mechanisms are responsible for phenotypic resistance.

Fluoroquinolones resistance in *Lactobacilli* has not been studied well, but in some studies such as Anisimova and Yarullina (2018), it had a high prevalence in investigated *Lactobacilli*, which shows the possibility of intrinsic resistance to these antibiotics. It is presumed that *Lactobacilli* are resistant to Trimethoprim, as they did not have the folic acid synthetic pathways (Charteris et al. 2001). In most studies such as Campdelli et al. (2018), Duskova et al. (2012), Trimethoprim-Sulfamethoxazole resistance has been reported as a prevalent phenotype in *Lactobacillus* isolates, but in this research, 20% of isolates (Nos. 3, 4, 14, 17, and 20) have shown resistance to this antibiotic (Anisimova and Yarullina, 2018; Temmerman et al. 2003).

Ciprofloxacin resistance, in this research, was prevalent (20.8% of isolates), including Nos. 2, 3, 8, 17, and 20; moreover, the results are in line with Erginkaya et al. (2018).

Vancomycin resistance is a typical phenotype in *Lactobacillus* and is often an inherent trait. In the present study, Vancomycin resistance (Dec et al. 2017) was the most common, and 11 of the isolates (45%), including Nos. 4, 5, 9, 10, 11, 12, 13, 14, 15, 16, and 17, were resistant to Vancomycin. Obtained results were in line with Campdelli et al. (2018), in which the Vancomycin-resistant phenotypes were the most common among isolates. The *van* (A) gene was found in seven isolates while 11 *Lactobacilli* were resistant to Vancomycin phenotypically, and the genotype and phenotype accordance was 63.6%.

In this research, all of the investigated *Lactobacilli* were susceptible to Rifampin obtained results conforming with Anisimova and Yarullina (2018) results and Campdelli et al. (2018). Multidrug-resistance is defined as resistance to at least one antibiotic in three or more antimicrobial groups according to ECDC criteria (Sukumarini et al. 2014) 12 isolates (60%) in this study were multidrug-resistant phenotypically, including isolates Nos. 1, 2, 3, 4, 8, 13, 14, 16, 17, 20, 22 and 23. Multidrug-resistance also was observed in Abriuel et al. (2015) study, which was 85%, and in Duskova et al. (2012) 17% of isolates were multidrug-resistant (Duskova et al. 2012). Multidrug-resistant isolates were present in this study, and the results are according to Abriuel et al. (2015) and Duskova et al. (2012), while in Belletti et al. (2009) study, multidrug-resistant phenotypes were not observed.

In conclusion, findings suggest that more studies are needed to investigate traditional and industrial starter cultures to reduce the risk of antibiotic resistance gene dissemination among symbiotic pathogens and normal flora. As isolates did not carry out resistance genes on their plasmid, it seems that they could be good candidates for more safety evaluation to introduce safe probiotic or starter culture strains.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Non-financial competing interests include (but are not limited to) political, personal, religious, ideological, academic, and intellectual competing interests.

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All authors have contributed to the design of the work, analysis of data and have drafted the work and substantively revised it. We have approved the submitted version (and any substantially modified version that involves the author's contribution to the study. Also, we have agreed to be personally accountable for the author's contributions and ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

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Figures

Figure 1
 Disk Diffusion Results for Lactobacillus isolated from Traditional Yogurt Using Ciprofloxacin, Penicillin, Gentamycin, Tetracycline, Vancomycin, as well as Erythromycin Antibiotic Disks on Muller Hinton Agar

Figure 2
 Shows Amplified PCR Products (1500 bp) from Six Extracted DNA, 100 – 10000 bp Markers Were Used. Isolates No. 16-21 in This Image Shows Positive Control, Which Is Lactobacillus. delbrueckii Subsp. Lactis PTCC.1743. (N) Is a Negative Control, for Lactobacillus Identification, 16S rDNA Primer Was Used, and Amplicon Sizes of PCR Products Were about 1500 bp

Figure 3

PCR Products Amplicons (168) bp from 7 Extracted DNA Isolates No. 5-11, 1000 bp Marker, P: Positive Control Tetracycline Resistance tet (W) Positive Lactobacilli tet w Found in One of them (number 8) N: Negative Control Which is Deionized Distilled Water

Figure 4

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Shows Amplified PCR Products (282) bp aad A Gene for Six Extracted chromosomal DNA, M: 1000 bp, (P) Positive Control is aadA Positive Lactobacilli and (N) Is Negative Control. (3, 4, 5, 6) which have shown in this figure.

Figure 5
Shows Amplified PCR Products van(A) Gene (730) bp, for 7 Plasmid Extracts of Lactobacilli, Including Isolates No. 12-18, (M) Is 1000 bp Marker,)P(Is Positive Control Which Is a van (A) Containing Lactobacillus Moreover,)N(is a Negative Control.