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ORIGINAL ARTICLES

Determination of some Antibiotic Resistances genes by Polymerase Chain Reaction of Lactic Acid Bacteria Isolates from Local Dairy Products

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ABSTRACT

Background: This study sheds light on the current resistance situation in local dairy product associated microorganisms Like Lactic Acid Bacteria (LAB) and will provide a basis for further experiments concerning gene transferability.

Objective: Isolation of Lactic Acid Bacteria (LAB) from local dairy products that have resistance phenotypes to antibiotics, and detection by PCR presence of certain known antibiotic resistance genes.

Materials and methods: This study was conducted during the period from 1st of September 2016 to the end of January 2017. Two hundred samples from local dairy products were collected as 50 samples from each raw milk, sour-milk, yogurt, cream and soft cheese. All isolates were identified according to biochemical characters, antimicrobial sensitivity test and MIC of the isolates was done by the Kirby–Bauer disc diffusion and micro dilution methods respectively. PCR amplification genes associated with resistance to β-lactam antibiotics (*bla* Z) gene, erythromycin (*erm*B), genes, and tetracycline ribosomal protection proteins (*tet* M), were done by PCR.

Results: The results showed that the most common bacteria presents in all dairy source samples were *Lactobacillus* as followed:75(31.9%), 40(17%), 30(12.7%) and 90(38.2%) in yogurt, white cheese, cream and sour milk respectively. *Lactococcus*

isolates was appeared as 20(36.3%) and 15(27.27%) in yogurt and creams respectively and 10(18.1%) in each of white cheese and sour milk. *Streptococcus* was found as 45(40.9%) in white cheese and 30(27.2%) in yogurt, while in cream and sour milk was found as 27(24.5%) and 8(7.2%) respectively, whereas the total number of *Lactococcus and Pediococcus* were 55(12.6%) and 36(8.25%) respectively. The physiological characters and phenotypic identification was found that out of the 436 LAB isolates; *Lactobacillus* isolates were the dominant genus appeared followed by *Streptococcus* species. Isolates of LAB demonstrated different profiles of antibiotic resistance, all *Lb. delbrukii*, *Lb. plantarum* and *Lb. fermentum* were resistance to penicillin G, while showed a variable susceptibility rates to other antibiotics. The detection of *tet* M and *erm* B and *bla* Z resistance genes in LAB isolates showed that some isolates harbor *tet* M and/or *erm* B and *bla* Z genes. Twenty isolates of *Lactobacillus delbrukii* showed the presence of *tet M* and *erm B* and 17 isolates harbor *bla* Z genes corresponding to their resistance phenotypes. As well as 10 isolates of *Lb. plantarum* showed these gens in some of them as follow; five of the ten possessed the resistance *tet M* gen and 4 isolates have *erm B* while 8 isolates showed the penicillin G resistance gen. From the ten isolates of *Lb. fermentum**;*** only 4 isolates harbor *tet M* gene and 5 isolates possessed *erm B* gens while 9 isolates had the resistance gens *bla Z.* The isolates *Pedicoccus sp. and Lc. lactis* showed only presence of *tet M* gens in 8 and 7 isolates respectively. On the other hand *Strep. thermophilus* (20 isolates); the band of the detected gens appeared in 18 isolates for *tet M* and 15 for the *erm B,* but a β-lactamase gene detected in only 4 of these resistant isolates. *Leu. mesntroids* (10 isolates) showed 6 and 4 isolates possessed *bla* Z and *tet M* gen respectively but only one isolates showed to possessing the gene *erm B.*

Conclusion: This study had established that wide varieties of LAB the most common bacteria presents in all local dairy source samples were lactobacillus species which show high resistance properties to amoxicillin and ampicillin. *Lactococcus lactis* isolates showed resistance to tetracycline and all *Leuconostoc cremoris* isolates showed sensitivity to all tested antibiotics except, some isolates resisted to tetracycline and chloramphenicol. While *Leuconostoc mesntroids* had variable resistance to tetracycline, chloramphenicol, erythromycin, vancomycin, amoxicillin, ampicillin and penicillin G. All *Streptococcus thermophilus* isolates were resisting to tetracycline. The genes *tet* M, *erm* B and *bla* Z were detected in Lactic acid bacterial isolates.

Key word: LAB, Antibiotics resistance, *tet M, erm B, bla Z* genes.

Introduction

Lactic acid bacteria (LAB) are a group of Gram positive bacteria and they yield lactic acid into the medium as a main fermentation product [1]. Many LAB species are present as contaminants on raw foods or intentionally added as starter cultures into them [2,3]. LAB have a long times past of safe use as fermenting natural products and probiotics intended for health benefits and have acquired the "Generally recognized as safe" (GRAS) status [4], but there is a great attention to these bacteria may serve as

reservoirs of antibiotic resistance[5]. LAB has been reported to be capable of supplying antimicrobial resistance genes to food-borne or enteric pathogens [6]. According to European Food Safety Authority [7], the presence of transmissible antibiotic resistance markers in these bacteria has become an important safety criterion. The greatest threat to the use of antimicrobial agents for therapy of bacterial infections has been the development of antimicrobial resistance in pathogenic bacteria. Shortly after the introduction of each new antimicrobial compound, emergence of antimicrobial resistance is observed [8]. The greatness of the problem is significantly increased by the possibility of bacteria to transfer resistance determinants horizontally and by the mounting increase in the use of antibiotics, which has created an enormous selective pressure towards resistant bacteria. Scott [9] concluded that gene transfer occurs widely in vivo between gastrointestinal tract bacteria, and pathogenic bacteria, as identical resistance genes are present in diverse bacterial species from different hosts. The evolution of antibiotic resistant food borne pathogens has been amply documented in recent years [10-12]. The food chain can be considered as the main route of transmission of antibiotic resistant bacteria between the animal and human population [13]. More specifically, fermented dairy products and fermented meats that are not heat-treated before consumption provide a vehicle for antibiotic resistant bacteria with a direct link between the animal indigenous microflora and the human gastrointestinal tract. Commercial introduction of probiotics containing antibiotic resistance strains may also have negative consequences, for example, when resistance is transferred to intestinal pathogens [14-16].

Materials and methods

Isolation and identification of LAB Isolates

Lactic acid bacteria were isolated from raw milk, sour-milk, yogurt, cream and soft cheese. A total of 200 local dairy products obtained from local markets in Erbil city, 25 gm. of each sample were collected and transferred in sterilized condition, within four hour; serial dilutions of homogenized samples were done, then cultured on selective solid media and incubated at 37 ºC. *Lactobacilli and Pediococci* was isolated under anaerobic conditions on MRS agar plates (Difco). *Lactococci* and *Streptococci* were cultured on M17 plates (Difco) under aerobic conditions. Up to 5 morphologically distinct colonies per plate were selected which characterized as rods or cocci by Gram stain and examined by light microscopy, were sub cultivated twice on the MRS and M17 agar plates to obtain pure cultures, for storage, liquid media over-night-cultures were frozen with 33% glycerol at -80ºC.

Identification of lactic acid bacteria

Identification of lactic acid bacteria, overnight cultures of each isolate in M17 and MRS broth were used. All isolates were initially tested for catalase and oxidase enzymes and production of acid from glucose and other sugars. Only Gram positive bacteria with catalase negative reactions were obtained according to Schillinger and Lucke [17]. For the growth at various temperatures 10, 15, 37 and 45°C, tolerance of different salt concentrations 2, 4 and 6.5% w/v NaCl were tested. The bacteria were

characterized by microscopic and by conventional biochemical and physiological tests. These preliminary tests make it possible to classify the isolates in genus on the basis of the characteristic and tests of identification as described by Dicks *et al.*, [18].

Determination of antibiotic susceptibility test

Antimicrobial susceptibility test of the isolates was done by the Kirby–Bauer disc diffusion method, according to Clinical Laboratory Standard Institute protocol [19]. Tests were performed with 9 discs (Oxoid) containing the following concentrations of antibiotics: 15 mg Erythromycin (Ery), 10 mg Amoxicillin (Amx), 10mg Ampicillin(Amp), 10 mg Penicillin G (Pen), 10 mg Streptomycin(Strep), 30 mg Tetracycline (Tet), 30 mg Vancomycin(Van), 30 mg Chloramphenicol (Chl), and 30 mg Cephalothine (Ceph). Bacterial colonies from fresh pure culture were mixed with peptone broth to prepare the turbidity of each inoculums was adjusted to McFarland 0.5 standards. Bacteria from each suspension were inoculated onto Muller Hinton agar using a sterile cotton-tipped swab. The plates were kept at 37°C for 10 min, to get them dry, before antibiotic discs were dispensed, then incubated in a microaerophilc atmosphere at 30°C for 48 h and the diameter of the inhibition zones was measured. The susceptibility patterns of the isolates were determined according to the National Committee for Clinical Laboratory Standard. Minimal Inhibitory Concentrations (MIC) for 3 antibiotics was 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-SensitestTM Broth (Oxoid) + 10% MRS Broth (Merck)+ 1,5% Agar [20] plates containing tetracycline, erythromycin and penicillin G antibiotics (Oxoid, Hampshire, UK) with the concentration range of 0.025-128 µg/ml . The MIC was defined as the lowest concentration of the antibiotic giving a complete inhibition of visible growth in comparison to an antibiotic free control point. Breakpoints were adopted from EFSA report [7, 21, 22].

Detection of antibiotic resistance genes in LAB isolates

Bacterial DNA extraction

DNA of bacteria isolates extracted according to Picozzi *et al.*, [23]; loop full of pure bacterial colonies was grown in 10 mL MRS broth for 18 hr. at 37 °C. A 500µl. of each culture was mixed with 500µL of cetyltrimethyl ammonium bromide (CTAB) buffer (50 mM hexadecyltrimethyl ammonium bromide, 1.4 mol L-1 NaCl 100 mmol L-1 Tris-HCl at pH 8.0, 20 mmol L-1 EDTA, 0.2%-mercaptoethanol), incubated at 65 °C for 30 min and then centrifuged at 12,000 *g* for 10 min. The supernatant was transferred to a new 1.5 ml Eppendorf tube, precipitated with one volume of isopropanol and centrifuged at 12,000 *g* for 10 min. After discarding the supernatant, the pellet was washed with 500 µL of 70% v/v ethanol before drying for 10 min. The pellet was dissolved in 100 µL. Tris-EDTA buffer (10 mmol L-1 Tris-HCl at pH 8.0, 1 mmol L-1 EDTA) and stored at -18 °C.

Detection of resistance genes by PCR

PCR amplification genes associated with resistance to β-lactam antibiotics *bla* (Z), erythromycin (macrolides) *erm* (B), genes, and tetracycline ribosomal protection

proteins *tet* (M), were detected in 50-µl volumes that contained 30 pmol of each specific primer, 1× Taq DNA polymerase buffer, each deoxynucleoside triphosphate at a concentration of 200 µM, 1 U of Taq DNA polymerase and 100 ng of genomic DNA used as a template. The oligonucleotide primers used included those, *erm* (B), *tet*(M), and the β-lactamase gene *bla*(Z) were used, which amplified PCR products as shown in Table 1. PCR-based detection of the *tet*(M,) gene was performed using the following thermal cycles: denaturation temperature 95 °C for 45 s, 52 °C for 45 s (25 cycles); While for *erm*(B) the thermal cycling program was as follows: 94 °C for 5 min; 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min (30 cycles), PCR amplification *bla* gene was done for 35 cycles at annealing temperatures 51°C for 1 min, and extension was done at 72°C for 45 s. Amplification products were detected by electrophoresis on a 1.8% agarose and subsequent staining with ethidium bromide solution.

Table 1: Primers, annealing temperatures for the detection of resistance genes

Resistance Gene	Primer	Annealing Temp.°C	Refer ence
tet(M)	**5'-GGTGAACATCATAGACACGC-3'** **5'-CTTGTTCGAGTTCCAATGC-3'**	**52°C**	**[24]**
erm(B)	**5'-GAAAAGGTACTCAACCAAATA-3'** **5'-AGTAACCGTACTTAAATTGTTTAC-3'**	**55 C**	**[25]**
bla(Z)	**5′- TACTTCAACACCTGCTGCTTTCG** **5′- ATTACACTCTTGGCGGTTTCAC -3′**	**51C**	**[26]**

Results

Isolation and identification of LAB

A total of 200 local dairy products samples collected from different markets; 436 isolates of LAB were obtained as follow: 235 isolates appeared on MRS agar, 110 isolates, isolated on M17 agar, as well as 91 isolates which isolated in MRS with cysteine and hydrochloride, Table 2. These three different media were used for isolation of lactic acid bacteria. Out of the 436 LAB isolates, the physiological characters phenotypic identification was found that Lactobacillus isolates were the dominant genus appeared on MRS (235, 53.8%) followed by *Streptococcus* species which isolated on M17 (110, 25.2%) and the number and percentage of the isolates appeared on MRS plus cysteine hydrochloride was 91(20.8%).

Table 2: Types of sources and number of positive samples of lactic acid bacteria

Types of sources	Number of collected samples	Number of the isolates on culture media		
		MRS	M17	MRS positive cysteine hydrochloride

Yogurt	**50**	**75**	**30**	**30**
White cheese	**50**	**40**	**45**	**20**
Creams	**50**	**30**	**27**	**22**
Sour milk	**50**	**90**	**8**	**19**
Total	**200**	**235(53.8%)**	**110(25.2%)**	**91(20.8%)**

The results presented in Table 3 showed that the most common bacteria presents in all dairy sources was *lactobacillus* as follow:75(31.9%), 40(17%), 30(12.7%) and 90(38.2%) in yogurt, white cheese, cream and sour milk respectively. *Lactococcus* isolates was appeared as 20(36.3%) and 15(27.27%) in yogurt and creams respectively and 10(18.1%) in each of white cheese and sour milk. On the other hand *Streptococcus* was found as 45(40.9%) in white cheese also 30(27.2%) in yogurt, while in cream and sour milk was found as 27(24.5%) and 8(7.2%) respectively, whereas the total number of *Lactococcus and Pediococcus* was 55(12.6%) and 36(8.25%) respectively as shown in Table 3 which illustrates the incidence of isolated LAB in relation to the different local dairy product samples

Table 3: Frequency of each type of lactic acid bacteria isolated from different sources of dairy products

Sample type	Sample number	Number (%)				Total LAB Isolates
		Lactobacillus spp.	*Lactococcus* spp.	*Streptococcus* spp.	*Pediococcus* spp.	
Yogurt	**50**	**75(31.9)**	**20(36.3)**	**30(27.2)**	**12(33.3)**	**137(31.4)**
White cheese	**50**	**40(17)**	**10(18.1)**	**45(40.9)**	**8(22.2)**	**103(23.6)**
Creams	**50**	**30(12.7)**	**15(27.27)**	**27(24.5)**	**10(27.7)**	**82(18.8)**
Sour milk	**50**	**90(38.2)**	**10(18.1)**	**8(7.2)**	**6(16.6)**	**114(26.1)**
Total	**200**	**235(53.8)**	**55(12.6)**	**110(25.2)**	**36(8.25)**	**436(100)**

Identification of isolated bacteria:

The isolates were identified according to biochemical, microscopic and cultural characteristics as illustrated in Table 4 based on the physiological characters, out of the total isolates obtained from different dairy products, showed Gram positive, non-motility, non- spore formation, not able to produce catalase, cocci which produce no gas from glucose. Among the cocci, some isolates were able to grow at 10 and 15, 37°C, and 45°C, as well as the isolates able to grow in 4%, but not in 6.5% NaCl concentration and not able to grew in 6.9 pH. The isolates formed acid from lactose and ribose but acid production from mannitol, sucrose and xylose was variable

between the isolates. These characteristics close resemblance to *Lactococcus lactis*. But the cocci isolates were able to grow at 10 and 45°C in 6.5% NaCl and pH 9.6 but not produce CO_2 from glucose were characterized as Enterococci seemed to be *Enterococcus faec*ium, as suggested by their ability to ferment sorbitol [27]. The isolates of Gram-positive rods grew at 15°C and did not form CO_2 from glucose. These characteristics suggest their classification as lactobacilli. Isolates of rods were classified as *Lactobacillus plantarum*, as suggested by their sugar fermentations patterns, all these isolates fermented the following sugar; arabinose, cellobiose, lactose, maltose, melibiose, raffinose, sucrose and trehalose, finally the other isolates were unable to ferment melibiose, raffinose, xylose, sucrose and arabinose classified as *Lactobacillus dellbrukii*. These species are frequently isolated from dairy products [28,29].

Table 4: Identification of lactic acid bacteria according to biochemical and physiological characters

Biochemical tests	***Lc. lactis***	***Ec. feacium***	***Streptococcus. thermophilus***	***Pediococcus sp.***	***Leu. cremorise***	***Lb. plantarum***	***Lb dellbrueckii***	***Lb. Fermentum***
Catalase	-	-	-	-	-	-	-	-
Oxidase	+	+	-	+	+	+	+	+
CO_2	+	+	+	+	+	+	+	-
Growth at								
10C°	+	+	+	+	+	+	+	+
15C°	+	+	+	+	+	+	+	+
37C°	+	+	+	+	+	+	+	+
45C°	_	+	+	+	+	+	+	-
NaCl 2%	+	+	+	+	+	+	+	+
2%	+	+	+	+	+	+	+	+
4%	+	+	+	+	+	+	+	+
6.5%	+	+	+	+	+	+	+	-
Acid from								
Glucose	+	+	-	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+
Sorbitol	-	+	-	-	+	+	+	+
Sucrose	+	+	+	-	+	+	+	+
Arabinose	-	+	+	-	-	+	-	-
Maltose	+	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	+	+	+
Mannitol	+	+	+	-	+	+	+	+

Xylose	+	-	-	-	-	+	-	-
Raffinose	-	-	-	-	-	+	-	-
Melebiose	-	-	-	-	+	+	-	-
Rammnose	-	-	-	-	-	-	+	-
Trehalose	+	+	-	-	-	+	_	+
Cellubiose	-	-	+	+	-	+	_	+

(+) Positive; (-) Negative

Sensitivity of the isolated LAB to antibiotics:

Tested isolates of LAB demonstrated different profiles of antibiotic resistance, as shown in Table 5. All *Lb. delbrukii, Lb. plantarum* and *Lb. fermentum* were resistance to penicillin G as well as showed more susceptibility to some antibiotics as follow; most *Lb. delbrukii i*solates 45(90%) and 41(82%)) were resist to amoxicillin and ampicillin respectively. While some isolates of *Lb. plantarum* and *Lb. fermentum*; 14(87.5%) and 16(100%); 12(85.7%) and 12(85.7%) were resist to amoxicillin and ampicillin respectively. Nearly high percentage 30(60%) of *Lb. delbrukii* isolates were resistant to cephalothine. All *Lactobacillus* species isolates were resistant to vancomycin and streptomycin, while *Lactobacillus* species showed moderate variation in the susceptibility to chloramphenicol, tetracycline, and erythromycin. All *Lctococcus lactis* (28%) isolates were sensitive to all tested antibiotics excluding 2(7.14%) of the isolates showed resistance to chloramphenicol and 20(71.42%) were resistant to tetracycline. *E. faecium* isolates show 44.4% resistance to chloramphenicol, while all the isolates showed (100%) sensitivity to other tested antibiotics. All *Leu. cremoris* showed sensitivity to all tested antibiotics except, 1(10%) and 3(30%) of the isolates were resistant tetracycline and chloramphenicol respectively. While *Leu. mesntroids* were sensitive to streptomycin and cephalothine, but they have variable resistance to tetracycline, chloramphenicol, erythromycin, vancomycin, amoxicillin, ampicillin and penicillin G. *Streptococcus thermophilus* isolates were susceptible to vancomycin, amoxicillin, ampicillin and cephalothine, but totally resist to tetracycline and low resistance of 2(4.5%), 4(9%), and 6(13.6%) demonstrated against chloramphenicol, penicillin G, and streptomycin respectively, and 40(90.9%) resistant to erythromycin. On the other side *Pediococci sp.* isolates were susceptible to erythromycin and amoxicillin and while with variable resistance to other tested antibiotics, Table 5.

Determination of the minimum inhibition concentration

The minimum inhibition concentration (MIC) of the highly resistances isolates of LAB to tetracycline, erythromycin and penicillin G was determined, Table 6. The results indicated that the MIC varied among the isolates, probably due to the extensive variability of resistance mechanisms conferring diverse levels of susceptibility specially with tetracycline [30].

All the isolates had a MIC value ranged between 0.01-1.5 µg/ ml for penicillin G, while with tetracycline the highest MIC observed was38 µg/ ml. and with

erythromycin the MIC ranged between 0.01-0.5 µg/ ml. As expected, the majority of the lactic acid bacteria isolates were highly penicillin G resistant, with the exception of *Pedicoccuc sp., Lac. lactis, Leu. cremorise* and *Enterococcus feasium* isolates. All *Streptococcus thermophillus* were resisting to tetracycline and 40 isolates resist to Erythromycin.

Detection of *tet, erm* and *bla* resistance genes by PCR

All of the resistance isolates of LAB to Tet, Ery and Pen. G were tested to detect the presences of these genes by PCR, the results showed that twenty isolates of *Lactobacillus delbrukii* which resists to tetracycline, erythromycin and penicillin G were tested to detect the presences of *tet M* and *erm B*, and *bla Z* genes by PCR ; the results showed that from the total (20) isolates showed the presence of *tet M* and *erm B* and 17 isolates harbor *bla Z* genes corresponding to their resistance phenotypes. Some isolates were positive for all genes or one or both genes, giving a 401-bp band for *tet M*, and a 405-bp band for *ermB* genes (Table 7 and Figures 1 A,B and C), as well as ten isolates of *Lb. plantarum* showed these gens in some of them as follow; five of the ten possessed the resistance *tet M* gen and 4 isolates have *ermB* while 8 isolates showed the pen G resistance gen. From the ten isolates of *Lb. fermentum;* only 4 isolates appeared the presences of *tet M* gene and 5 isolates possessed *erm B* gens while 9 isolates had the resistance gens *bla* Z, the isolates *Pediococcus sp. Lactococcus Lactis* showed only presence of *tet M* gens as 8 and 7 isolates respectively. On the other hand *Strep. thermophilus*(20); the band of the detected gens appeared in 18 isolates for *tet M* and 15 for the *erm B*, but a β-lactamase gene could be detected in only 4 of these resistant isolates with specific *bla* Z gene primers. *Leu. mesntroids* showed 6 and 4 isolates possessed *bla Z* and *tet M* gen respectively but only one isolates showed to possessing the gen *erm B* as shown in Table 7.

Table 5: The Susceptibility of Isolated Bacteria to Antibiotics

Type (No. of isolates)	Number and Percentage(%) of resistance antibiotics								
	Tet	Chl	Ery	Van	Amx.	Amp.	Penc	Strep	Ceph
Lb. dellbruekii (50)	19 (38)	15 (30)	30 (60)	50 (100)	45 (90)	41 (82)	50 (100)	50 (100)	30 (60)
Lb. plantarum (16)	8 (50)	10 (62.5)	11 (68.75)	16 (100)	14 (87.5)	16 (100)	16 (100)	16 (100)	8 (50)
Lb. fermentum (14)	6 (42.85)	4 (28.57)	8 (57.1)	14 (100)	12 (85.7)	12 (85.7)	14 (100)	14 (100)	5 (35.71)
Ped. sp. (10)	8 (80)	1 (10)	0	2 (20)	0	1 (10)	1 (10)	7 (70)	4 (40)
Strep.	44	2	40	0	0	0	4	6	0

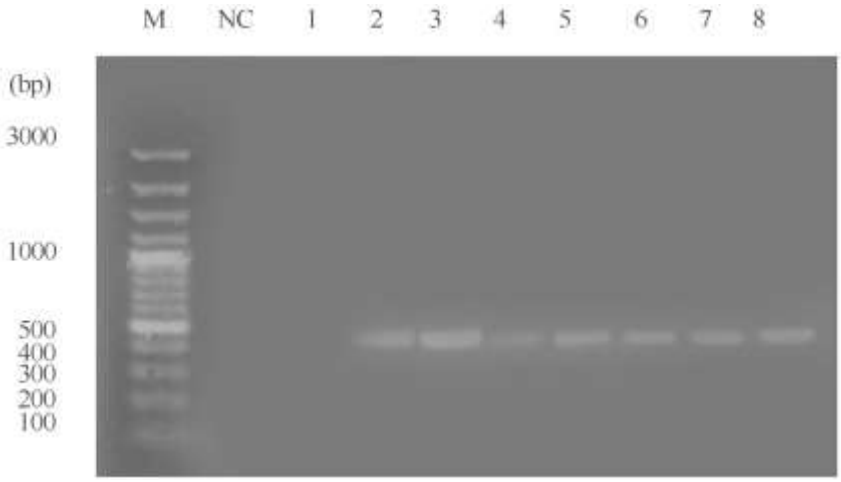
Thermophiles **(44)**	**(100)**	**(4.5)**	**(90.9)**				**(9)**	**(13.6)**	
Lc. lactis **(28)**	**20 (71.42)**	**2 (7.14)**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
E. faecium **(18)**	**0**	**8 (44.4)**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Leu. cremoris **(10)**	**1 (10)**	**3 (30)**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Leu. mesntroids **(18)**	**4 (25)**	**5 (27.7)**	**2 (11)**	**1 (5.5)**	**4 (22.2)**	**6 (33.3)**	**8 (55.5)**	**0**	**0**

Table 6: MIC of resistant LAB isolates

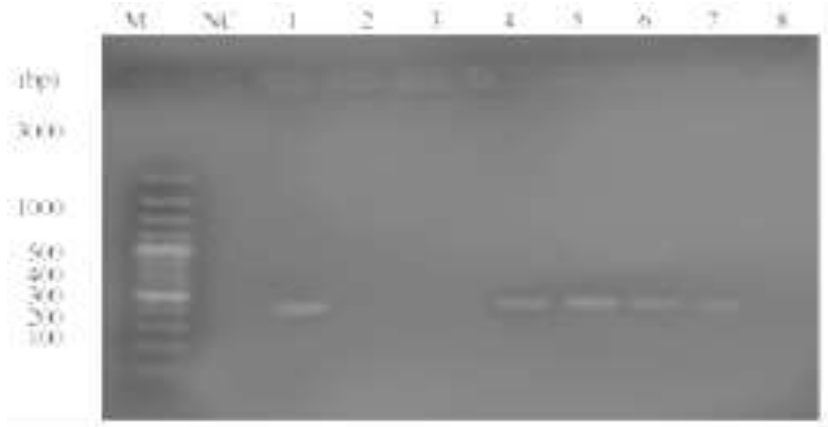
Bacteria (No. of The isolates)	**MIC in µ/ml**		
	Tetracycline	**Erythromycin**	**Penicillin G**
Lb. dellbruekii **(20)**	**4-34**	**0.5-1**	**0.25-0.5**
Lb. plantarum **(10)**	**4-38**	**0.5-1.25**	**>0.25- 0.5**
Lb. fermentum **(10)**	**4-34**	**0.5-1.25**	**0.25- 0.5**
Ped. sp. **(10)**	**2- 12**	**>0.025**	**0.125-0.25**
Strep. thermophilus**(20)**	**4-8**	**0.25-0.5**	**0.01-0.05**
Lc. lactis **(10)**	**2-8**	**0.01-0.02**	**0.025**
E. faecium **(10)**	**4-32**	**>0.025**	**0.01-0.02**
Leu. cremoris **(10)**	**8-16**	**>0.5**	**0.5-1.5**
Leu. mesntroids **(10)**	**4-24**	**0.0125-0.25**	**0.02- 0.05**

Table 7: Detection of Resistance Gens (*tet M, ermB and bla)* by PCR

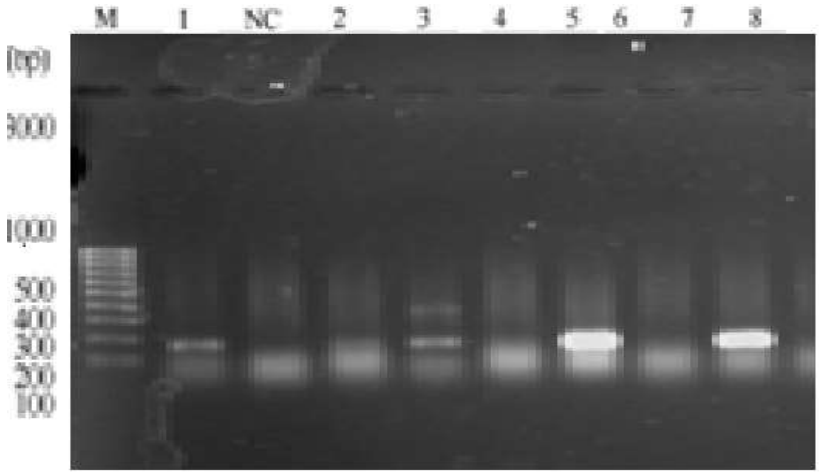
Bacteria	**Number of positive isolates**		
(No. of The isolates)	***tet M gene***	***ermB gene***	***bla* gene**
Lb. dellbruekii **(20)**	**20**	**20**	**17**
Lb. plantarum **(10)**	**5**	**4**	**8**
b. fermentum **(10)**	**4**	**5**	**9**
Ped. sp. **(10)**	**8**	**-**	**-**
Strep. thermophilus**(20)**	**18**	**15**	**4**
Lc. lactis **(10)**	**7**	**-**	**-**
Leu. mesntroids(**(10)**	**4**	**1**	**6**



A



B



C

Figure 2.A - PCR detection of *tet*(M) resistance gene in some *Lactobacillus* spp. Lane M: 3000-bp marker, lane NC: Negative control, lane 2-8: 401-bp band of *tet*(M) gene, lane 1: no bands with DNA [no *tet*(M) gene]. B – *erm B* resistance gene in *Lactobacillus* spp. Lane M: 3000-bp marker, Lane NC: Negative control, Lane 1, 4, 5, 6 and 7: 405-bp band of *ermB* gene, Lane 2, 3 and 8: no bands with DNA (no *erm*B gene). C- *bla* Z resistance gene, lane M:3000 bp marker, lane N; negative control, lane1,4,6,8,11; 325 bp band of *bla* Z gene

Discussion

In the present study, 436 LAB isolates from local dairy products were isolated and identified by biochemical tests. After bacterial identification, the isolates were evaluated by phenotypic and genotypic methods for their antibiotic resistance profiles. Phenotypic assays that used to determine the antibiotic susceptible/resistant patterns have been complemented by molecular methods in which bacterial isolates are directly screened for the presence of antibiotic resistance determinants. This study revealed to use PCR for detection *tet* M and *erm* B and *bla* resistance genes in LAB isolates. It was found that some isolates harbor *tet* M and/or *erm* B and *bla* genes and others that were previously showed tetracycline or erythromycin resistant patterns, were found to be negative for *tet* M or *erm* B genes respectively. These false results can be explained by the fact that there is currently no standard method for antibiotic susceptibility testing of LAB, although several micro dilution methods have been used. Also, many factors may affect the susceptibility results such as the inoculum size, the incubation time, the incubation temperature, the composition of the atmosphere and the growth medium. An increased inoculum size and an extended incubation time resulted in elevated antibiotic MICs for some species [31].

Nawaz *et al.* [32], reported that out of 84 LAB strains, *erm* B gene was detected in eight *Lactobacillus* strains and one *Streptococcus thermophilus* strain. The *tet* genes were identified in 12 strains of lactobacilli from traditional foods which is consistent with our results. Also, detection of *tet* M and *erm* B resistance genes have been previously investigated [33,34]. The resistance of the studied *Lactobacillus* spp. to antibiotics was variable according to species and antibiotic tested, some *Lb. fermentum* isolates were detected as resistant to tetracycline and *tet* M gene was found in some of these isolates as a resistance gene. only one *Lb. fermentum* was resistant to chloramphenicol. D'Aimo *et al*., [35], Bujnakova *et al*., [36], they were illustrated that *Lactobacillus* species are generally susceptible to chloramphenicol, erythromycin and tetracycline. On the other hand Gfeller *et al*.,[37], Cataloluk and Gogebakan [38], reported that six isolates of *Lactobacillus casei* and *Lactobacillus fermentum* isolates from dairy products and human origin carried *tet*(M) genes. Kastner *et al.* [39], studied 161 LAB isolates for antibiotic resistance, and only one *Lb. reuteri* strain (SD 2112) showed a high tetracycline resistance phenotype that could be correlated with a *tet*(W) resistance gene. Erythromycin resistance genes have been reported to occur on conjugative plasmids in lactobacilli such as plasmid pGT633 from *Lb. reuteri* strain 100-63, or pLEM3 from *L. fermentum* LEM89 [40].

In the present study, 436 LAB isolates from local dairy products were isolated and identified by biochemical tests. After bacterial identification, the isolates were evaluated by phenotypic and genotypic methods for their antibiotic resistance profiles. Among the Pediococci isolates, only some were resist to tetracycline and *tet*(M) gene was detected in eight resistant *Pediococcus sp isolates*. Ammor *et al*. [41], indicated the antibiotic susceptibility of *Pediococcus* spp. isolated from food was very rare. Hummel *et al*. [42], investigated antibiotic resistances of 45 lactic acid bacteria those belong to the genera *Lactobacillus, Streptococcus, Lactococcus, Pediococcus* and *Leuconostoc*. One of the *Pediococcus* strain *Ped. pentosaceus* strain BFE 7436 was found to show low resistance to tetracycline. However, it was reported that neither of the genes those encode the ribosomal protection proteins [*tet*(M), *te*t(Q), *tet*(S) or *tet*(W)] nor the genes those encode the tetracycline efflux pumps [*tet*(K) or *tet*(L)] were found Tetracycline resistance seems to be common in *P. sp* isolates in our study, tet M gene could be detected in many of these resistant strains. It has been reported by Gevers *et al*. [43]; Shalini and Rameshwar [44]; Sabir *et al*. [45] that the antibiotic tetracycline usually an active against *Pediococcus pentosaceus* isolates and susceptibility levels are thought to be species-dependent [42]. *Enterococcus faecium* strains isolated in our study were found to be resistant to different antibiotics. *En. faecium isolates* showed high level of resistance to Erythromycin (32 µg/ml), and also Chloramphenicol resistant which was isolated from dairy products but none of these isolates , *erm*(B) or bla genes could be detected. One of the all *En. faecium isolates* were resistant to tetracycline and carried *tet*(M) gene.

In a study undertaken by Temmerman *et al.* [46], a total of 29 *En. faecium* strains were isolated from different European probiotic products and antibiotic

resistance was detected against tetracycline (24% of the isolates), Erythromycin (97% of the isolates) and Chloramphenicol (34% of the isolates). The resistance of *Enterococcus* species isolated from Turkish white cheese samples to 13 antibiotics is studied by Citak et al., [47], and 96% of *En. faecium* isolates were found to be resistant to Erythromycin, whereas 76% and 44% were resistant to Chloramphenicol and Tetracycline respectively. Huys *et al.* [48] have found that 24% of *Enterococcus* isolates from European cheeses displayed phenotypic resistance to Tetracycline with MIC ranges of 16 to 256 μg/ml. *En. faecium* GLM-160 and GLM-161 were resistant to Ciprofloxacin with MIC values of 4 μg/ml. Similarly, Ciprofloxacin resistance has been described among *En. faecium* isolates from different food sources at varying degrees [49, 50].

Conclusion: This study had established that wide varieties of LAB the most common bacteria presents in all local dairy source samples were lactobacillus in Erbil city and lactobacilli are considered to be one of the most important potential Accurate characterization and identification of LAB and the exact screening for the presence of antibiotic resistance determinants requires the combined use of phenotypic properties and molecular methods. Lactobacilli species from dairy products show high resistance properties to Amoxicillin and Ampicillin but showed moderate variation in the susceptibility to Chloramphenicol, Tetracycline and Erythromycin. *Lactococcus lactis* isolates appeared resistance to Tetracycline also all *Leuconostoc. cremoris* isolates showed sensitivity to all tested antibiotics except, some isolates resisted to Tetracycilin and Chloramphenicol respectively, while *Leuconostoc mesntroids* had variable resistance to Tetracyclin, Chloramphenicol, Erythromycin, Vancomycin, Amoxicillin, Ampicillin and Penicillin G. All *Streptococcus thermphilus* isolates were resist to Nalidixic acid and Tetracyclin and low-level were resistance to Chloramphenicol, Penicillin G and Streptomycin, antibiotics and the *tet* (M), *erm* (B) and *bla* (Z) genes were detected in Lactic acid bacteria isolates. This is attributed to the stricter quality control measures and the proper characterization and maintenance of starter culture strains during the production of local dairy products**.**

References

1. Schleifer KH, Ludwig W. Phylogenetic relationships of lactic acid bacteria. In: Wood BJB, Holzapfel WH. Blackie Academic And Professional, Glasgow,1995; Pp. 7 – 18.
2. Capcarová M, Weis J, Hrnčár C, Kolesárová A, Petruška P, Kalafová A, Pál G. Effect of probiotic supplementation on selected indices of energy profile and antioxidant status of chickens. J Microbiol Biotech Food Sci 2011; 1 (2) 225-235.
3. Sharma N, Garcha S, Singh S. Potential of *Lactococcus lactis* Subsp. lactis Mtcc 3041 as a bio preservative. J Microbiol Biotech Food Sci 2013; 3 (2) 168-171
4. Mathur S, Singh R. Antibiotic resistance in food lactic acid bacteria. Int J Food Microbiol 2005;105 281-295

5. Thumu S, Halami P. Acquired resistance to macrolide- lincosamide-streptogramin antibiotics in lactic acid bacteria of food origin. Indian J Microbiol 2012; 52 (4) 530-537.
6. Gevers D, Huys G, Devlieghere F, Uyttendaele M, Debeverf J, Swings J. Isolation and identification of tetracycline resistant lactic acid bacteria from pre-packed sliced meat products. Syst Appl Microbiol 2000; 23: 279-284.
7. EFSA. Technical guidance update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance prepared by the Panel on Additives and Products or Substances used in Animal Feed. European Food Safety Authorization 2008; 732: 1-15
8. Levy SB. Antibiotic resistance: an ecological imbalance. In: Chadwick DJ, Good J. Antibiotic resistance, origins, evolution, selection and spread. John Wiley & Sons, Chichester,1997; Pp. 1– 14.
9. Scott KP. The Role of conjugative transposons in spreading antibiotic resistance between bacteria that inhabit the gastrointestinal tract. Cell. Mol. Life. Sci.2002; 59: 2071–2082.
10. Teuber M, Perreten V. Role of milk and meat products as vehicles for antibiotic-resistant bacteria. Acta Vet. Sci and Suppl 2002; 93: 75– 87.
11. Threlfall EJ, Ward LR, Frost JA, Willshaw GA. The emergence and spread of antibiotic resistance in food-borne bacteria. Int. J. Food Microbiol 2000; 62: 1 –5.
12. White DG, Zhao S, Simjee S, Wagner DD, Mcdermott PF. Antimicrobial resistance of foodborne pathogens. microbes Infect 2002; 4: 405– 41
13. Witte W. Impact of antibiotic use in animal feeding on resistance of bacterial pathogens in humans. In: Chadwick, DJ, Goode J. antibiotic resistance: origins, evolution, selection and spread, foundation symposium. Wiley, Chichester 2008; Pp. 61–75
14. Gamal Fadl M, Gad Ahmed M, Zeinab Shawky H Farag. Antibiotic resistance in lactic acid bacteria isolated from some pharmaceutical and dairy products. Brazilian Journal Of Microbiology 2014; 45(1) 25-33
15. Tajudeen AB, Bolanle AA, Muinah JF. Antibiotic resistance patterns of lactic acid bacteria isolated from Nigerian grown salad vegetables Afr. J. Microbiol. Res.2017; 11(11) 433-439.
16. Atia AE, Ashour A, Abired A. Survey on knowledge towards antibiotics among medical university students in Libya. Int J Medi Pharm Res 2018; 4(2) 61-66.
17. Schillinger U, and Lucke FK. Identification of Lactobacilli from meat and meat product. Food Microbiol 1987; 4: 199-208
18. Dicks LM, Fantuzzi L, Gonzales FC, Toit MD, Dellaglio M. *Leuconostoc argentinum* isolated from argentine raw milk. Int J Syst Bacteriol 1993; 43: 347-351.
19. Clinical and Laboratory Standards Institute (CLSI) Performance standards for antimicrobial susceptibility testing.2012; 22^{sd} Ed. Wayne.
20. Iso 10932. The International Organization for Standardization. Milk and milk products — determination of the minimal inhibitory concentration of antibiotics applicable to bifido bacteria and non-enterococcal Lactic Acid Bacteria (LAB) 2010; part 2.
21. Ouoba Lii, Lei V, Jensen LB. Resistance of potential probiotic lactic acid bacteria and bifido bacteria of African and European origin to antimicrobials: determination and transferability of the resistance genes to other bacteria. Int J Food Microbiol 2008; 121: 217-224.

22. Danielsen M, Wind A. Susceptibility of *Lactobacillus* Spp. to antimicrobial agents. Int J Food Microbiol 2003; 82: 1-11.
23. Picozzi C, D'anchise F, Foschino R. PCR detection of *Lactobacillus Sanfranciscensis i*n sourdough and pantone baked product. Eur Food Res Technol 2003; 222:330-35.
24. Werner G, Willems RJ, Hildebrandt B, Klare I, Witte W. Influence of transferable genetic determinants on the outcome of typing methods commonly used for *Enterococcus faecium*. J Clin Microbiol 2003; 41: 1499- 1506.
25. Sutcliffe J, Grebe T, Tait-Kamradt A, Wondrack L. Detection of erythromycin-resistant determinants by pcr. Antimicrob Agents Chemotherapy 1996; 40 (11) 2562-2566.
26. Chang Liu, Zhuo-Yang Zhang, Ke Dong, Jian-Ping Yuan, and Xiao-Kui Guop. Antibiotic resistance of probiotic strains of lactic acid bacteria isolated from marketed foods and drugs. Biomedical and Environmental Sciences 2009; 22: 401-412.
27. Manero A and Blanch AR. Identification of *Enterococcus* ssp. with a biochemical key. Applied Environ. Microbiol 1999; 65: 4425-4430
28. Huiling Guo, Lin Pan, Lina Li Jie, Lu Laiyu, Kwok Bilige, Menghe Heping, Zhang Wenyi Zhang. Characterization of antibiotic resistance genes from *Lactobacillus* isolated from traditional dairy products. J. Food Science 2017; 82(3) 724-730
29. Egervärn M, Lindmark H, Roos S, Huys G, Lindgren S. Effects of Inoculum size and incubation time on broth microdilution susceptibility testing of lactic acid bacteria. Antimicrob Agents Chemother 2007; 51:394–396
30. Devirgiliis 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 2010; 109:313–323.
31. Toomey N, Bolton D, Fanning S. Characterization and transferability of antibiotic resistance genes from lactic acid bacteria isolated from Irish pork and beef abattoirs. Research in Microbiology.2010;161:127–135.
32. Nawaz M, Wang J, Zhou A, Ma C, Wu X, Moore JE, Millar BC, Xu J. Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. Curr Microbiol 2011; 62:1081–1089.
33. Ayeni FA, Sánchez B, Adeniyi BA, Los Reyes-Gavilán CG, Margolles A, Madiedo PR. Evaluation of the functional potential of Weissella and Lactobacillus isolates obtained from Nigerian traditional fermented foods and cow's intestine. Int J Food Microbiol 2011; 147: 97-104.
34. Cataloluk O, Gogebakan B. Presence of drug resistance in intestinal Lactobacilli of dairy and human origin In Turkey. Fems Microbiol Lett 2004; 236: 7-12.
35. D'aimmo M R, Modesto M, Biavati B. Antibiotic resistance of Lactic Acid Bacteria and *Bifidobacterium spp*. isolated from dairy and pharmaceutical products. Int J Food Microbiol 2007; 115 (1) 35- 42.

36. Bujnakova D, Strakova E, Kmet V. *In vitro* evaluation of the safety and probiotic properties of lactobacilli isolated from chicken and calves. Anaerobe 2014; 29: 118-127.
37. Gfeller KY, Roth M, Meile L, Teuber M. Sequence and genetic organization of the 19.3-kb erythromycin- and dalfopristin resistance plasmid plme300 from *Lactobacillus fermentum* rot1. Plasmid 2003; 50: 190–201.
38. Cataloluk O, Gogebakan B. Presence of drug resistance in intestinal lactobacilli of dairy and human origin in Turkey. Fems Microbiol Lett 2004; 236: 7-12.
39. Kastner S, Perreten V, Bleuler H, Hugenschmidt G, Lacroix C and Meile L. Antibiotic susceptibility patterns and resistance genes of starter cultures and probiotic bacteria used in food. Syst Appl Microbiol 2006*;* 29**:**145-*155.*
40. Teuber M, Meile L and Schwarz F. Acquired antibiotic resistance in lactic acid bacteria from food. Antonie Leeuwenhoek 1999;*76:135*-155.
41. Ammor M, Flórez A, Mayo B. Antibiotic resistance in non-enterococcal lactic acid bacteria and bifido bacteria. Food Microbiol 2007*;*24: 559-70.
42. Hummel AS, Hertel C, Holzapfel WH, Franz CM. Antibiotic resistances of starter and probiotic strains of Lactic Acid Bacteria. Appl Environ Microbiol 2007*;*73: 730-739.
43. Gevers D, Huys G, Swings J. *In vitro* conjugal transfer of tetracycline resistance from lactobacillus isolates to other gram-positive bacteria. Fems Microbiol. Lett. 2003; 225: 125– 130.
44. Shalini M, Rameshwar S. Antibiotic resistance in food lactic acid bacteria - A review. International Journal of Food Microbiology 105(3):281-95.
45. Sabir F, Beyatli Y, Cokmus C, Onal-Darilmaz D. Assessment of potential probiotic properties of *Lactobacillus Spp*., *Lactococcus Spp*. and *Pediococcus Spp*. strains isolated from Kefir. J Food Sci 2010;75 (9) 568- 573
46. Temmerman R, Pot R, Huys B, Swings J. Identification and antibiotic susceptibility of bacterial isolates from probiotic products. Int J Food Microbiol 2003; 81(1) 1-10.
47. Citak S, Yücel N, Orhan S. Antibiotic resistance and incidence of *enterococcus* species in Turkish white cheese. Int J Dairy Technol 2004;57 (1) 27-32.
48. Huys G, D'haene K, Collard JM, Swings J. Prevalence and molecular characterization of tetracycline resistance in *enterococcus* isolates from food. Appl Environ Microbiol 2004;70: 1555-1562.
49. Valenzuela AV, Omar NB, Abriouel H, Lopez RL, Ortega E, Canamero MM, Galvez A. Risk factors in enterococci isolated from foods in morocco: determination of antimicrobial resistance and incidence of virulence traits. Food Chem 2008; 2648–2652.
50. Başbülbül Melihcan Özteber, Haci Halil Biyik. Antibiotic resistance in Lactic Acid Bacteria isolated from fermented dairy products and boza. J Microbiol Biotech Food Sci 2015; 4 (6) 513-517