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Molecular Investigation of *Lactobacillus plantarum* isolated from Raw cow milk in Kirkuk/Iraq

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Abstract

Lactobacillus plantarum is one of the most popular lactic acid bacteria that is widely applied in the food industry as a probiotic and natural preservative. Thus, this study was designed to isolate *Lactobacillus plantarum* from raw cow milk from Kirkuk city and to identify the strains phenotypically and by molecular means. Accordingly, five *Lactobacillus plantarum- like* strains were isolated out of sixty milk samples. These strains were identified phenotypically as white, round colonies when grown on MRS medium. They were Gram-positive rods, non-motile and catalase-negative. In addition, those organisms showed strong resistance to some antibiotics. On the other hand, the isolates showed sensitivity to other β-lactam agents which included ampicillin and ceftriaxone. Furthermore, the isolates showed strong antibacterial activity against some indicator bacteria such as *Staphylococcus aureus* and *Staphylococcus epidermidis* and then against the pathogenic bacteria *E.coli* and *Pseudomonas aeruginosa.*

Additionally, the taxonomy of the isolates was recognized to the species level by partial sequencing of the 16S rRNA gene and the analysis of the phylogenetic tree. Consequently, the five isolated strains showed 99% homology with some *Lactobacillus plantarum strains* listed in the NCBI. Afterwards, the isolates were registered in the NCBI.

Keywords: *Lactobacillus plantarum*, Raw cow milk, Molecular characterization, Kirkuk, Iraq.

Introduction

Lactic acid bacteria are anaerobic- aerotolerant Gram-positive diverse groups of bacteria that have been widely used in the food industry (Axelsson and Ahrné; Ray and Joshi; Park *et al* .,2016) though, Lactobacilli have been extensively used for decades in medicine for their probiotic potential (Bernet *et al* ., 1994; Tannock, 1999). As well, the *Lactobacillus plantarum* species is one of the most popular *Lactobacilli* known to produce various antibacterial substances such as bacteriocins (Sgouras and Maragkoudakis, 2004; Klaenhammer, 1998; Garneau *et al* ., 2002). Those bacteriocins are natural antimicrobial peptides with bactericidal activity and a high commercial value since it is produced from species of bacteria that are “generally recognized as safe” (GRAS) and has a qualified presumption of safety status (QPS) (Koutsoumanis *et al* ., 2020; Koutsoumanis *et al* ., 2021). However, despite the fact of the high ability of the *Lactobacillus plantarum* survival and adherence to the epithelial cells of the gastrointestinal tract and its safety to humans according to (FAO and WHO); it has five closely taxonomic species that could not be distinguished by the conventional phenotypic methods (Behera *et al* ., 2018). Recently, several molecular screening methods were developed for the comparison between the subspecies of the *Lactobacillus plantarum*.

Therefore, this study aimed to isolate *Lactobacillus plantarum* from local cow milk samples and characterize them to the molecular level along to the phylogenetic tree of the isolated strains.

Methods:

1. Collection of Milk samples

Sixty milk samples were collected from local cow breeds from the Laylan sub-district in Kirkuk/Iraq during the period from December 2020 to February 2021. Milk samples were collected daily at seven o'clock in the morning in clean sterile containers, then were immediately transferred to the microbiology laboratory of Biology Department /College of Education for Pure Science**/** Kirkuk University according to the methodology cited in (Kumar and Murugalatha, 2012).

2. Isolation and primary characterization of the isolates

Collected milk samples were diluted in peptone medium and were incubated at 37℃ for 30 minutes; then, one millilitre of diluted milk samples spread on de Man, Rogosa, Sharpe (MRS) selective medium and plates were incubated at 37℃ for 48 hrs with 5-10% CO_2 according to (Ahirwar *et al* ., 2017).

3. Identification of the *Lactobacillus* isolates

Colonies growing on de Man, Rogosa, Sharpe (MRS) medium were identified according to their morphological, cultural, Gram's staining and biochemical properties which included; Catalase, oxidase tests, Arginine hydrolysis, Indole test, Citrate utilization and carbohydrate fermentation tests and growth at 15℃ and 45℃. Results were compared to the growth pattern of Lactobacilli species mentioned in Bergey's Manual of Systematic Bacteriology cited by (Tawfeeq and Abbas, 2020).

4. Antibiotic sensitivity of the *Lactobacillus* isolates

Susceptibility of the isolates to eight types of antibiotics was performed by the disc diffusion method as described by (Bauer *et al* ., 1966).) using commercially available antibiotics disc (Bioanalyze/Turkey) containing; Amikacin (10 µg), Cefteriaxon (10 µg), Ciprofloxacin (10 µg), Gentamicin (10 µg), Cefotaxime (30 µg), Ampicillin (25 µg), Ceftazidime (30 µg**)** and Amoxicillin/clavulanic acid (30 µg). Antibiotic discs were placed on the surface of the agar plates cultivated with the *Lactobacillus* isolates. The plates were then incubated at 37 °C for 24 h and inhibition zone diameters were measured. Results were expressed as sensitive, S (≥ 21 mm); intermediate, I (16-20 mm) and resistant, R (≤ 15 mm).

5. Evaluation of bacteriocin production and activity

Isolates were grown on MRS broth at 37℃ for 48hrs, then culture supernatant was obtained by centrifugation at 12.000xg for 10 minutes at 4℃. The antibacterial activity was determined by the agar diffusion assay cited by (Liasi *et al* ., 2009) where aliquots of the supernatants (100 µL) were placed in (6mm/d) wells on plates seeded with (1%v/v) of two gram-positive bacteria as indicator strain(*Staphylococcus aureus and Staphylococcus epidermidis*) then the activity tested against two other pathogenic bacteria *Ecoli* and *pseudomonas aeruginosa*. Plates were incubated at 37℃ for 48hrs and growth inhibition zones were recorded.

6. Molecular identification of the isolates by 16s rRNA gene sequence

6.1- Amplification of16s rRNA gene sequence

The genomic DNA of the selected isolates was extracted using the (G-spin™ Genomic DNA Extraction Kit/1NtRON/ Korea) according to the company instructions; then the quantity of the DNA was determined by Quantus fluorometer.
Afterwards, the gene sequence was amplified with a thermal cycler using one set of bacterial universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG- 3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') and products were analyzed with electrophoresis by running the samples on 1.5% agarose gel according to (Qian *et al* ., 2018).

6.2- DNA sequencing & phylogenetic tree analysis

Polymerase chain reaction products were sent for Sanger sequencing by Macrogen/Korea and results were analyzed with Geniuos software. Subsequent taxonomy of the isolates was determined through a phylogenetic tree followed by registration of the isolates in the NCBI according to the methodology cited in (Hajigholizadeh *et al* ., 2020).

Results & Discussion

1- Isolation and identification of *Lactobacillus* isolates

A total of sixty milk samples were collected aseptically from local cow breeds from the Laylan district. All samples were serially diluted and cultivated on MRS selective medium incubated at 37℃ for 24-48 hrs.
Single bacterial colonies grown on MRS medium plates were re-cultivated on the same medium and pure cultures were subjected to standard procedures for the identification of *Lactobacillus* isolates. The morphological, biochemical and sugar fermentation test results showed the isolation of five isolates of *Lactobacillus plantarum* like strains displayed in figure (1).

Figure 1: (a) Distinctive colony characteristics of the *Lactobacillus plantarum* isolates grown on MRS agar medium for 48hrs at 37°C and 1-10%CO_2.

(b) Gram's-stained *Lactobacillus plantarum* cells viewed under 100X of the light microscope.

As shown in figure 1b, the isolated strains reacted positively with Gram's stain and bacterial cells appeared as long rods under a light microscope. They did not possess flagella nor created endospores. In addition, purified cultures of the isolates were able to grow optimally at pH between 5.5 and 6.5 when grown in MRS broth at 37°C. Also, the isolates did not reduce nitrates nor liquified gelatin and were not indole producers. Their catalase test was negative and these results agreed with the results obtained by (Tajabadi *et al* 2013) upon the identification of *Lactobacillus plantarum* isolated from fermented sausages in Brazil (Tajabadi *et al* 2013).

Furthermore, isolates were subjected to antibiotic sensitivity tests and results showed different patterns in antibiotic resistance and sensitivities as table 1 showed.

Table1: Assessment of resistance and sensitivity patterns of five strains of Lactobacillus isolated from raw cow milk in Kirkuk/Iraq.

AMC	AK	AM	CN	CAZ	CTX	CIP	CRO	Isolate No
S	S	S	S	R	S	S	S	1
S	R	S	R	R	R	R	S	2
R	R	S	R	R	R	R	S	3
R	R	S	R	R	S	R	S	4
R	S	S	R	R	R	R	R	5

It could be noticed from the table above that, the five isolates were resistant to the aminoglycoside antibiotic (Amikacin and Gentamicin) and Quinolones (Ciprofloxacin). On the other hand and despite the absolute resistance to Ceftazidime four isolates were sensitive to the" β- lactam" antibiotic Ceftriaxone while the five isolates were absolutely sensitive to (Ampicillin). These results agreed with the results obtained by (Liasi *et al* 2009; Alias *et al* ., 2019).

However, the isolates were furtherly tested for their antibacterial activity against two indicator bacteria(*Staphylococcus aureus* and *Staphylococcus epidermidis*) (Ming *et al* ., 2015) and two pathogenic bacteria (*Pseudomonas aeruginosa* and *E. coli*) isolated from different clinical samples and used as indicator organisms; results were displayed in table 2.

Table 2: Antimicrobial activity of the isolated *Lactobacillus* strains against some pathogenic bacteria.

Indicator bacteria	*Lactobacillus* isolates No.	Antimicrobial activity	Degree of inhibition

Streptococcus epidermidis	LB1-5	+++	Strong ≈ 5mm
Staphylococcus aureus		+++	Strong ≈ 5mm
Pseudomonas aeruginosa		++	Moderate ≈ 3mm
E. coli		-	No activity

However, the results obtained in Table2 agreed with the results obtained by (Le *et al* ., 2019; Lei *et al* ., 2020; Hassan *et al* ., 2020) where they indicated the antibacterial activity of the Bacteriocin produced from their isolates as an antibacterial agent. The same was counted with the isolates of the present study; where they showed no activity against *E. coli* since the latter is a bacteriocin producer by itself according to (Refay *et al* .,2020).

Consequently, the five isolates were investigated by molecular means and genomic DNA was extracted and amplified with PCR using two universal primers and the products were analyzed using 1.5% agarose gel electrophoresis and results of 16S rRNA gene amplification showed a DNA fragment with the expected length of (1350 bp) per sequence. The same result was obtained by (Saeed *et al* 2020) who isolated the same bacteria from goat raw milk in Basrah/Iraq (Saeed *et al* 2020).

Accordingly, the results of sequencing and phylogenetic analysis showed that the five *Lactobacillus* isolates had about 99 % homology with identified *Lactiplantibacillus plantarum* recorded in the GenBank database of NCBI. as shown in Figure (2) and (3)

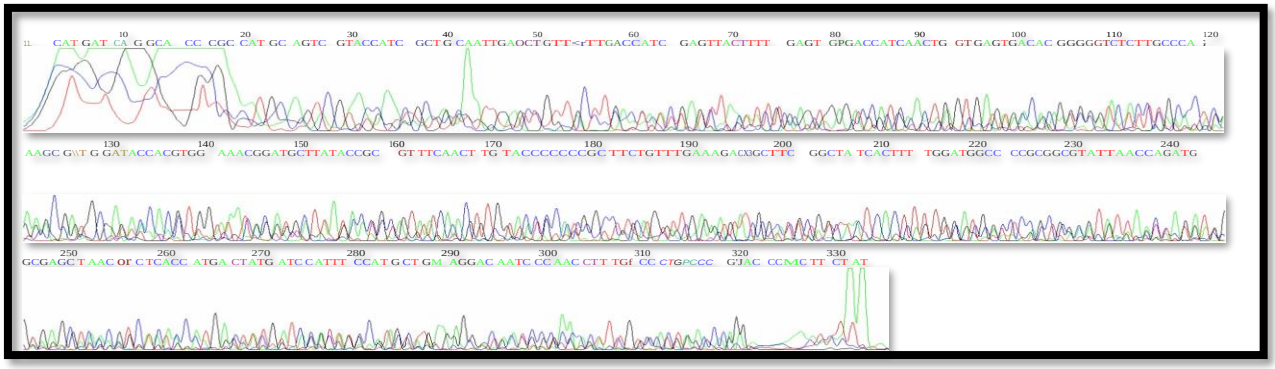

Figure 2: 16S rRNA partial gene sequence of *Lactobacillus plantarum* WSAK1 (MZ156858.1) Iraq 2021

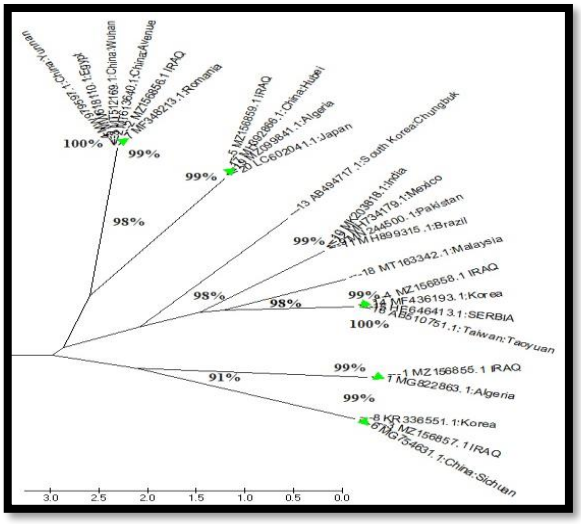

Figure 3: phylogenetic tree showing the phylogenetic placement of *Lactiplantibacillus plantarum* WSAK1 (MZ156858.1) Iraq 2021 as inferred by the neighbor-joining tree method based on 16S rRNA partial gene sequence analyses.

However, the strains showed similarity with other strains from some different countries across the world listed in NCBI as shown in Table 3

Table 3: The identification and homology results of five isolates of Lactiplantibacillus plantarum by 16S rRNA partial gene sequence

	Accession	Country	Source	Compatibility
1.	ID: MG822863.1	Algeria	Lactiplantibacillus plantarum	99%
2.	ID: MT613640.1	China	Lactiplantibacillus plantarum	99%
3.	ID: MT512169.1	China: Wuhan	Lactiplantibacillus plantarum	99%
4.	ID: MW979597.1	China: Yunnan	Lactiplantibacillus plantarum	99%
5.	ID: MW618110.1	Egypt	Lactiplantibacillus plantarum	99%
6.	ID: MG754631.1	China: Sichuan	Lactiplantibacillus plantarum	99%
7.	ID: MF348213.1	Romania	Lactiplantibacillus plantarum	99%
8.	ID: KR336551.1	Korea	Lactiplantibacillus plantarum	99%
9.	ID: MN244500.1	Pakistan	Lactiplantibacillus plantarum	99%
10.	ID: MK203818.1	India	Lactiplantibacillus plantarum	99%
11.	ID: MH899315.1	Brazil	Lactiplantibacillus plantarum	99%
12.	ID: MH734179.1	Mexico	Lactiplantibacillus plantarum	99%
13.	ID: AB494717.1	South Korea	Lactiplantibacillus plantarum	99%
14.	ID: MF436193.1	Korea	Lactiplantibacillus plantarum	99%
15.	ID: HE646413.1	SERBIA	Lactiplantibacillus plantarum	99%
16.	ID: AB510751.1	Taiwan	Lactiplantibacillus plantarum	98%
17.	ID: MH392866.1	China:	Lactiplantibacillus plantarum	97%

18.	ID: MT163342.1	Malaysia	Lactiplantibacillus plantarum	99%
19.	ID: MZ099841.1	Algeria	Lactiplantibacillus plantarum	99%
20.	ID: LC602041.1	Japan	Lactiplantibacillus plantarum	99%

The same results were obtained with the *Lactiplantibacillus plantarum* isolated by (Barbosa *et al* in 2021) from Arugula in Portugal (Barbosa *et al* ., 2021).

Then, the identified strains were registered at the GenBank database of the NCBI through direct submission and their accession number is listed in table 4 below.

Table 4: The accession number of the registered *Lactiplantibacillus plantarum* isolates in the NCBI

Isolate no.	Isolate name	accession number in NCBI	Gene Length/bp
1	*Lactobacillus plantarum* WSAK1	MZ156855.1	277
2	*Lactobacillus plantarum* WSAK2	MZ156856.1	805
3	*Lactobacillus plantarum* WSAK3	MZ156857.1	699
4	*Lactobacillus plantarum* WSAK4	MZ156858.1	980
5	*Lactobacillus plantarum* WSAK5	MZ156859.1	836

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