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Investigation of the DNA Sequencing of Some Iraqi Local Strains of Homofermentative Lactic Acid Bacteria

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Abstract
Lactic acid bacteria (LAB) are one of most useful bacteria found in milk. Four isolates have been isolated in this study, and identified by biochemical and physiological tests. By molecular identification, one of these isolates has been changed in to another genus throughout DNA sequencing, and another supposed being as new world strain.
Keywords: Lactococcus, Enterococcus, Lactic acid.

1. Introduction

Lactic acid bacteria are divided into two major groups: The homofermentative group, which produces lactic acid as the sole product of the fermentation of sugars, and the heterofermentative, which besides lactic acid also produce ethanol, as well as CO_2. The difference between the two is as a result of the absence of the enzyme aldolase in the heterofermenters. Aldolase is a key enzyme in the E-M-P pathway and spits hexose glucose into three-sugar moieties (Okafor 2007). In homofermentative lactic acid bacteria, Hutkins (2006) demonstrated that the NADH formed during the glyceraldehyde-3-phosphate dehydrogenase reaction must be re-oxidized by lactate dehydrogenase, so that the [NADH]/[NAD] balance is maintained, which include *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *L. delbrueckii* subsp. *bulgaricus* (used as dairy starter organisms); *Pediococcus* sp. (used in sausage cultures); and *Tetragenococcus* (used in soy sauces), while in heterofermentative the oxidation of NADH and maintenance of the [NADH]/[NAD_] balance occurs via the two reductive reactions catalyzed by acetaldehyde dehydrogenase and alcohol dehydrogenase, which included *L. mesenteroides* subsp. *cremoris* and *Leuconostoc lactis* (used in dairy fermentations), *L. mesenteroides* subsp. *mesenteroides* and *Leuconostoc kimchii* (used in fermented vegetables), *Oenococcus oeni* (used in wine fermentations), and *Lactobacillus sanfranciscensis* (used in sourdough bread).

The family Enterococcaceae is circumscribed on the basis of phylogenetic analyses of the 16S rRNA sequences and includes the genus *Enterococcus*, *Melissococcus*, *Tetragenococcus*, and *Vagococcus* (Ludwig *et al*., 2009). Normal habitat is the intestinal contents of humans, animals, and birds, and the environment. Can establish on equipment surfaces. Used as an indicator of sanitation. Important in food spoilage (Ray 2005). One of the important enterococci is *Enterococcus faecium*, because in addition to lactic acid production, has the ability to produce antimicrobial agents; Badarinath & Halami (2011) found that the native isolate of *E. faecium* MTCC 5153 can produce anti listerial enterocin that can be effectively used to reduce the risk of *Listeria, Staphyllococcus* and enterococci based food spoilage and contamination, and this culture is an useful source for the enterocin A biosynthesis gene.

Teuber (2009) indicated that *Lactococcus* is a member of the family *Streptococcaceae*, along with *Streptococcus* and *Lactovum*, in the order *Lactobacillales*. *Lactococcus lactis* is the most widely used lactic acid bacterium. *L. lactis* is used for the production of cheese, butter, buttermilk, and other fermented milks and, to some extent, is used in meat, bread, and vegetable products (Hansen 2004). Due to nucleic acid hybridization studies and immunological relationships of superoxide dismutase it was transferred by (Schleifer *et al*. 1985) from the old name *Streptococcus lactis* to its recent name. *Lactococcus lactis* has the ability to produce antimicrobial agents also, (Badr *et al*. 2005) isolated nisin from *Lactococcus lactis* Fc2 with molecular weight of3.5 kDa and after storage for two weeks it had a molecular weight of7 kDa.

This study has aimed for isolating of some homofermentative lactic acid bacteria, and identifying them with biochemical and physiological tests in addition to the molecular characterization by DNA sequencing for identifying to the level of strain.

2. Material and methods

2.1. Isolation and cultivation of bacteria.

The media M17 broth (Himedia, India) and de mann Rogosa Sharpe (MRS) agar (Oxoid, England) have been prepared according to the instructions of manufacturing companies as possible, with addition of approximately 1.5- 2g /100 ml agar to M17 broth for making M17 agar. All media have been sterilized by autoclave adjusted at 121°C, 15- 20 min. and 0.15 Mpa. A loopful of raw animal milk sample have been cultured on M17 and/ or MRS plates, left in the incubator adjusted at 30°C± 1for 24- 48 or 72 hr. according to (Bulut 2003) with modifications.

Different colonies have been sub cultured by streaking for purification (Grainger *et al*. 2001).

2.2. Morphological characterization.
Description on plates for colonies has been characterized on M17 and MRS according to (Bulut 2003) with modifications by recognizing of shape, color, constancy and size. Whereas Gram staining (Arcomex, Jorden) has been applied for selected isolate by following the manufacturing company information as possible.

2.3. Physiological identification.
The tests have been applied according to (Al- Asady 2003), with some modifications, as the following: Growth at temperatures of 10 and 45°C, growth at NaCl concentration (4 and 6.5%) and growth at pH 9.6.

2.4. Biochemical tests.
According to Harisha (2007), Bulut (2003) and Harrigan & MacCans, (1976) catalase test slide technique, clotting test and gas production test have been done respectively with some alterations, while Strepto- system 9R (Liofilchem, Italy) test has been processed by following the instruction of manufacturing as possible.

2.5. Genetic identification.
2.5.1. Genomic DNA extraction
Cultures of isolates have been activated on M17 broth for 18- 24 hr., centrifuged at 7000- 8000 cycle/min. for 10-15 min., washed with distilled water at the same as preparing to the procedure of the genomic DNA mini kit (Geneaid, Taiwan) as possible, with some changes in concentrations and time for the addition materials.
2.5.2. Detection of genomic DNA by gel electrophoresis
Method of electrophoresis by using 0.8% agarose has been done according to Sambrook and Russell (2001) as possible due to facilities.
2.5.3. Identification by Polymerase Chain Reaction (PCR).
Primers for 16SrDNA B27F (5-AGAGTTTGATCCTGGC-3) and U1492R (5-GGTTACCTTG TTACGACTT-3) are used (Lane, 1991), along with PCR program selected from Miyoshi *et al*. (2005), adapted by Hussein (2013) table (2. 1). The amplification steps abstracted from Prokić *et al*. (2009) with little changes.

Table (2. 1): PCR amplification program.

Step	Temperature	Time	No. of cycle
Initial denaturation	92 °C	2 min	1
Denaturation	94 °C	30 sec	30
Annealing	51.8 °C	45 sec	
Extension	72 °C	1.5 min	
Final extension	72 °C	5 min	1

2.5.4. Detection of 16S rDNA by gel electrophoresis.
Method of electrophoresis by using 2% agarose has been done according to Sambrook & Russell (2001) in order to detect the 16S rDNA gene, with some differences required.
2.5.5. Purification, sequencing and manipulation of data for PCR product.
The obtained product of PCR has been sent for Bioneer Company laboratories/ Korea, for purification and sequencing. Then data have been manipulated by assist. Lecturer Khloud AbdulKareem/ College of Nursing.

3. Results.
3.1. Isolation and cultivation.
Suspected isolates have been selected from raw animal milk samples termed as B1, B2, B3 and B5.

3.2. Morphological characterization.
3.2.1. Colonies characteristics.
On M17 and MRS agar, isolates were white, creamy, pin point to small colonies after 24hr.
3.2.2. Gram staining.
Staining has shown that isolates are Gram positive, cocci or ovoid in pairs or chain, as in the figures (3. 1a, b, c and d).

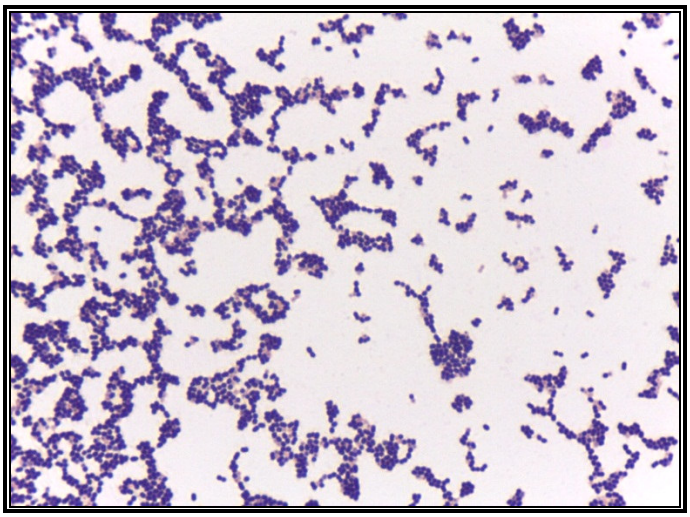

Figure (3. 1a): Gram staining of B1.

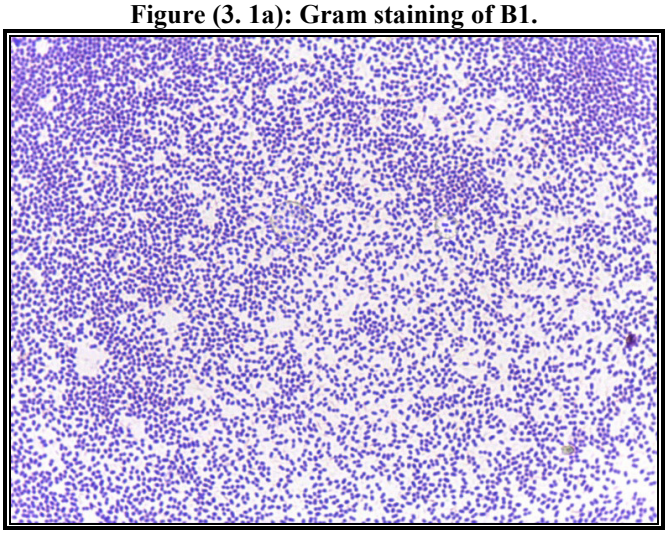

Figure (3. 1b): Gram staining of B2.

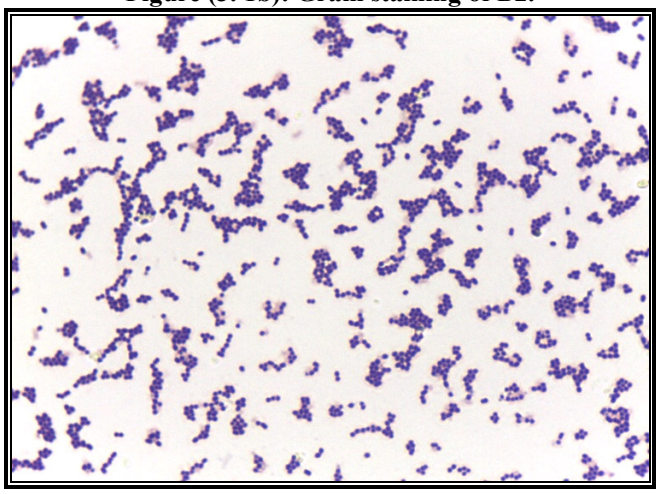

Figure (3. 1c): Gram staining of B3.

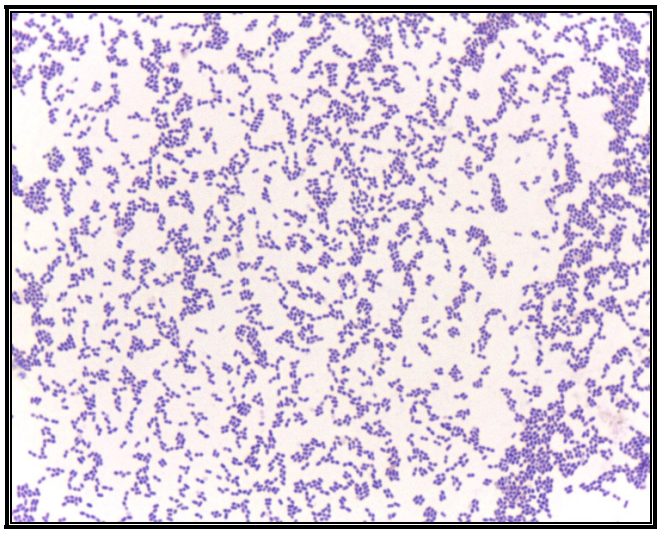

Figure (3. 1d): Gram staining of B5.

3.3. Biochemical tests.

Isolates were negative to the catalase and no bubbles have been shown and in the skim milk, clot has been produced in all tubes in contrast with control, besides; no gas has been liberated neither in M17 nor MRS broth from the isolates. In addition, different reactions have been shown in the system Strepto- system 9R tests, as found in the table (3. 1).

Table (3. 1): Strepto- system 9R test

Isolate / Test	B1	B2	B3	B5
Pyroglutamic-β-Naphthylamide	+	-	+	-
Aesculin	+	V	+	V
Hippurate	-	-	-	V
O-Nitrophenyl-Beta-D-Galactopiranoside	+	+	+	V
Arabinose	-	-	-	-
Mannitol	-	+	-	+
Raffinose	-	-	-	-
Bacitracin	-	-	-	-
Optochine	-	-	-	-

; + positive, - negative, V variable

3.4. Physiological identification.

Study of growth under different temperatures, NaCl and pH has been compared in table (3. 2).

Table (3. 2): Growth of isolates under different conditions;

Growth condition / Isolate	Temperature(ºC)		Salinity(NaCl%)		pH
	10	45	4	6.5	9.6
B1	+	+	+	+	+
B2	+	V	+	+	+
B3	+	+	+	+	+
B5	+	V	+	+	+

3.5. Genetic identification.

3.5.1. Genomic DNA extraction and detection.

Electrophoresis technique has revealed clear isolated DNA for the all isolates as found in the figures (3.3a and b).

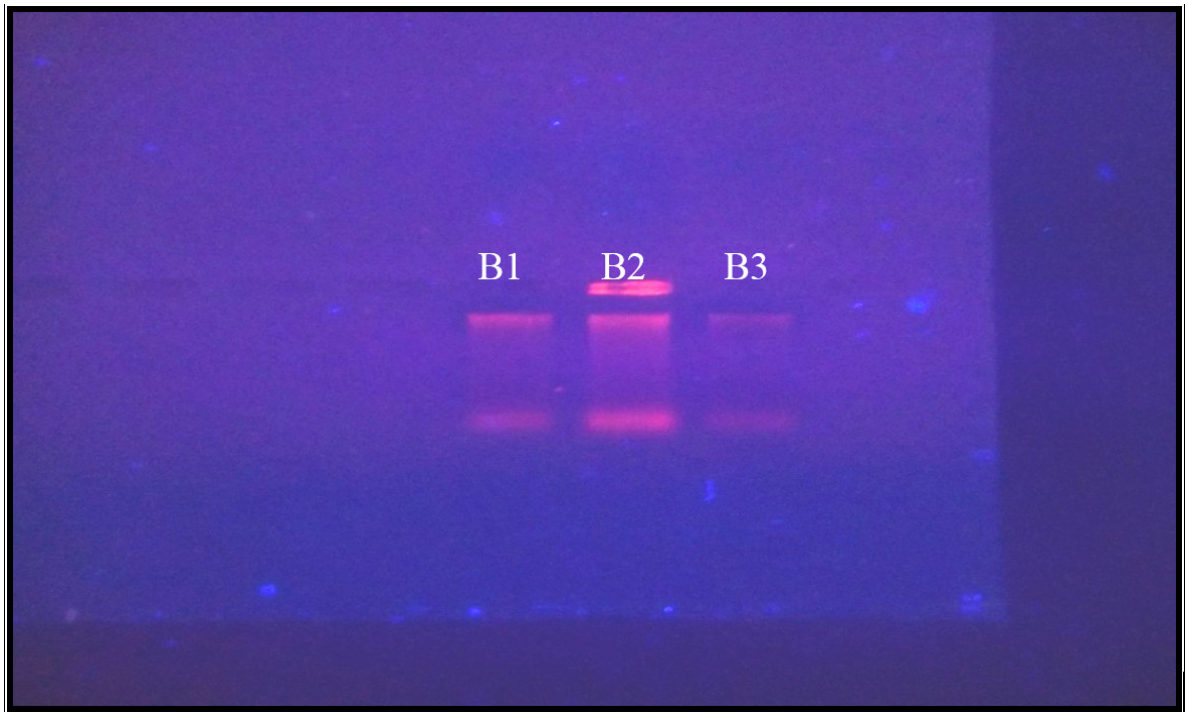

Figure (3. 3a): Gel electrophoresis for the isolated genomic DNA; (B1- B3): number of isolates.

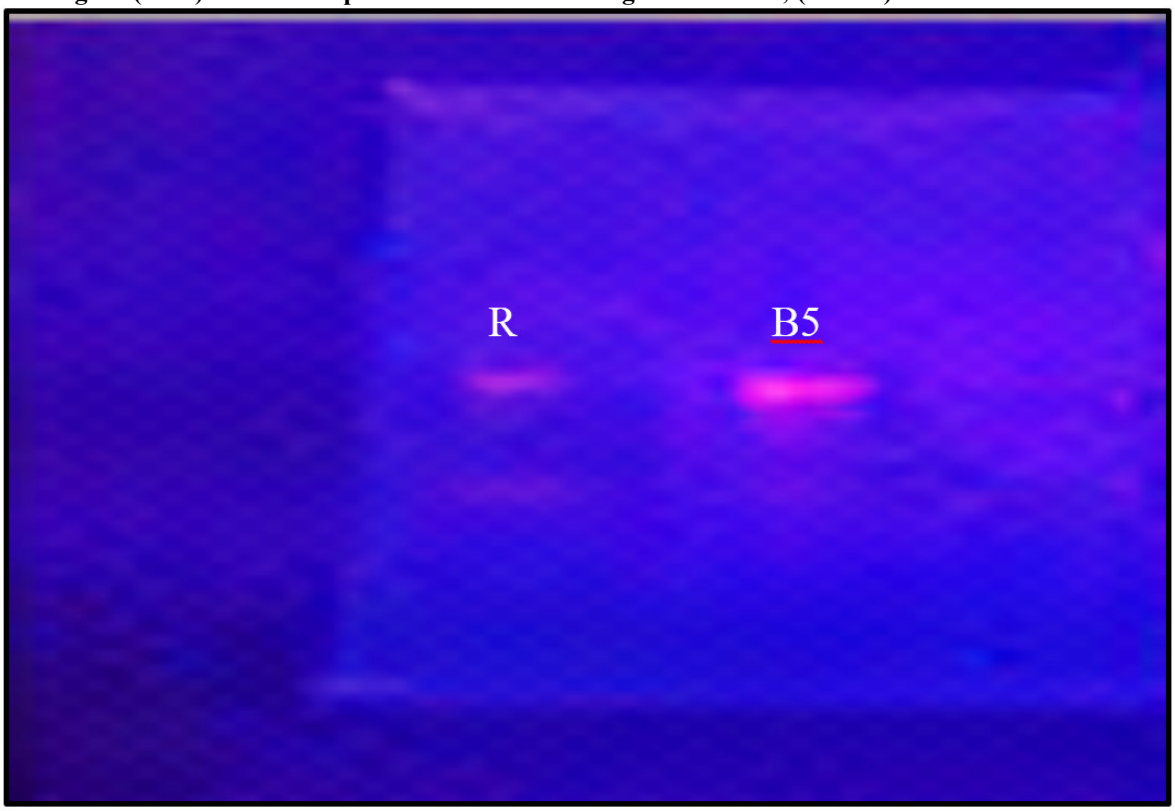

Figure (3. 3b): Gel electrophoresis for the isolated genomic DNA of the isolates; B5: number of isolate. R: reference strain (*Streptococcus thermophilus* from yogurt).

Amplifying of the 16S rDNA gene by (PCR) Technique.

By using a universal primer, results have obtained the required band of 16S rDNA for each isolates along with electrophoresed ladder in the region of 1500bp, as in the figure (3. 4a and b).

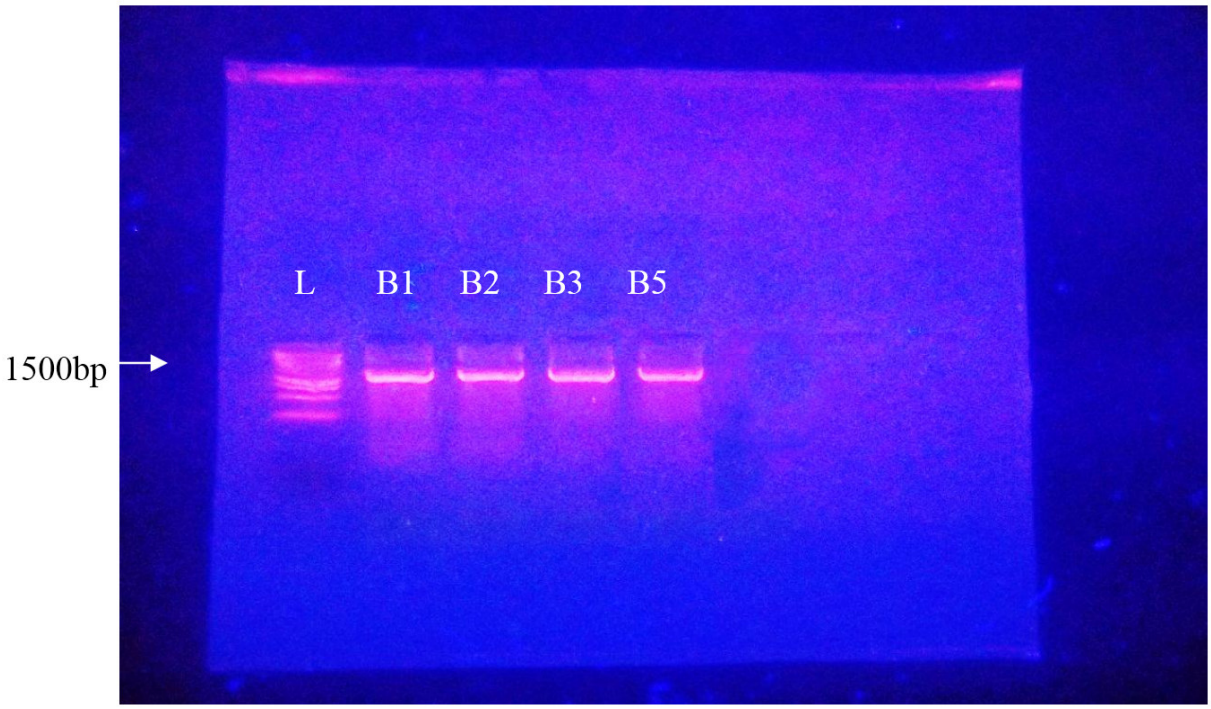

Figure (3. 4a): Gel electrophoresis for PCR technique; (B1- B5): number of isolates, L: DNA ladder of 1Kb, :16S rDNA gene 1500bp.

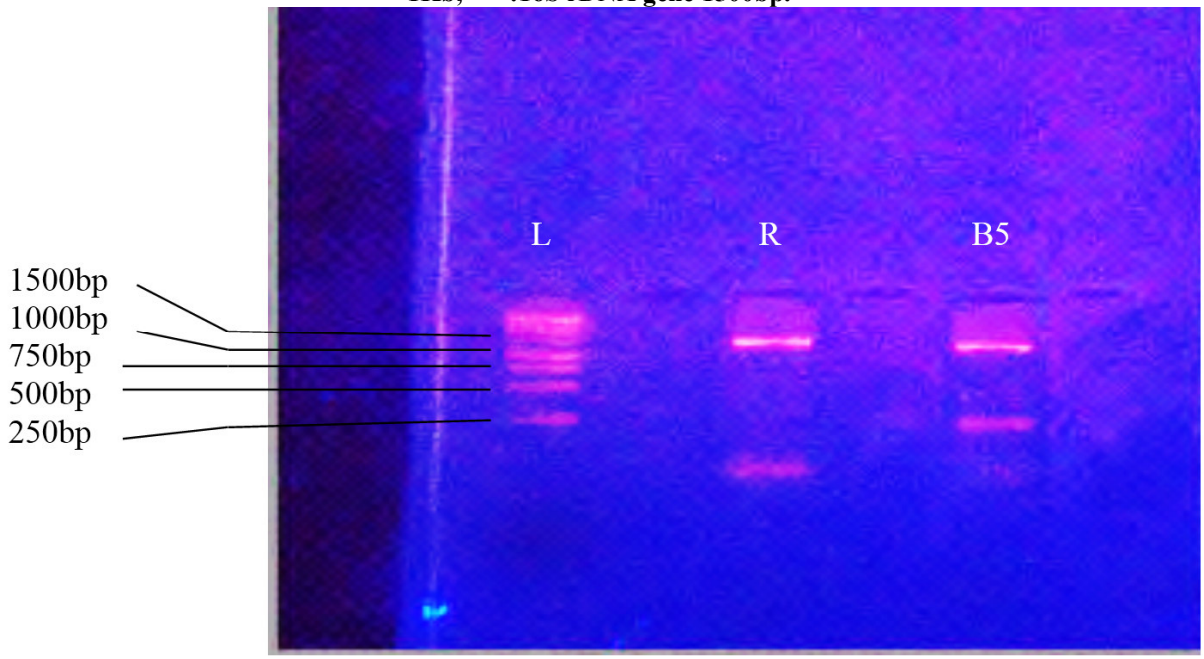

Figure (3. 4b): Gel electrophoresis for PCR technique; (B5): number of isolates, L: DNA ladder of 1Kb, R: reference strain (*Streptococcus thermophilus* from yogurt), (Repeated figure for more opening ladder).

Sequencing

The four isolates have been identified to the level of strain, as in the table (3. 3).

Table (3. 3): Sequencing of isolates after data manipulation

Isolate	Sequence	Identity (%)	Original Strain	Length (bp)
B1	GCTCCACCGGAAAAAGAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAG GGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAATCAAAACCGCAGGTTTTGATTTGAAA GGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTC ACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCC AAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGC CGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTA ACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA ATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGT CTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAG AGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAA GGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATAC CCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAG CTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCaAAGGAATTGACGGG GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGaAGAACCTTACCAGGTCTTG ACATCCTTTGACcACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGgtGGTGCATGGTTG TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTT	99	*Enterococcus faecium* IMAU94247	1024
B2	GGAGTAACGCGTGGGGAATCTGCCTTTGAGCGGGGGACAACATTTGGAAACGAATGCTAATACCGC ATAAAAACTTTAAACACAAGTTTTAAGTTTGAAAGATGCAATTGCATCACTCAAAGATGATCCCGC GTTGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGATGATACATAGCCGACCTGAGAGGG TGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTC GGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACT CTGTTGGTAGAGAAGAACGTTGGTGAGAGTGGAAAGCTCATCAAGTGACGGTAACTACCCAGAAAG GGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTG GGCGTAAAGCGAGCGCAGGTGGTTTATTAAGTCTGGTGTAAAAGGCAGTGGCTCAACCATTGTATG CATTGGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGC GTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCCTGTAACTGACACTGAGGCTCG AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGAT GTAGGGAGCTATAAGTTCTCTGTATCGCAGCTAACGCAATAAGCACTCCGCCTGGGGAGTACGACC GCAAgGTTGAAACTCAAaGGAATTGACGGGGGCCCGCACaAGCGGTGGAGCATGTGGTTTAATTCGA AGCAACGCGAaGaACCTTACCAGgTCTTGACATACTCGTGCTATTCCTAGAGATAgGAAGTTCCTTCG GGACACGGGATACAgGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG CAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTAAGTTGGGCACTCTAACGAGACTGCCGGTGA TAAACCGGAGGAAGGTGGGGATGACGTCAAATCAAAGCGGCTCTCTGGCCTGTAACTGACACTGAG GCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGC TAGATGTAGGGAGCTATAAGTTCTCTGTATCGCAGCTAACGCAATAAGCACTCCGCCTGGGGAGTA CGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA ATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATACTCGTGCTATTCCTAGAGTCATGCCCC TTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACAGTGATGTTTAG CTAATCTCTTAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCG CTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAC ACCACGGGAGTTGGGAGTACCCGAAGTAGGTTG	100	*Lactococcus lactis* subsp. *lactis* TW35	1094
B3	AACTGGATGAGCAGCGAACGGGTGAGTAACGCGTGGGGAATCTGCCTTTGAGCGGGGGACAACATT TGGAAACGAATGCTAATACCGCATAAAAACTTTAAACACAAGTTTTAAGTTTGAAAGATGCAATTG CATCACTCAAAGATGATCCCGCGTTGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGATG ATACATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG GAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAA GAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGAAGAACGTTGGTGAGAGTGGAAAGCTCATCA AGTGACGGTAACTACCCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTC CCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTATTAAGTCTGGTGTAAAA GGCAGTGGCTCAACCATTGTATGCATTGGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAA TTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGG CCTGTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC GCCGTAACGATGAGTGCTAGATGTAGGGAGCTATAAGTTCTCTGTATCGCAGCTAACGCAATAAGC ACTCCGCCTGGGGAGTACGACCGCAAGgTTGAAACTCAAaGGAaTTGACGGGGgCCCGCACAaGCGG TGGAGCATGTGGTTTAATTCGAAGCAACGCGAGAaCCTTACCAGgTCTtGACATACTCGTGCTATTcCT AGAGATAGGAAGtCCTTCGGGACACGGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTAAGTTGGGCAC TCTAACGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTA TGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACAGTGATGTTTAGCTA ATCTCTTAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTA GTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACC ACGGGAGTTGGGAGTACCCGA	100	*Lactococcus lactis* subsp. *lactis* Vm214	1347
B5	GCAGTTGAGCGCTGAAGGTTGGTACTTGTACCAACTGGATGAGCAGCGAACGGGTGAGTAACGCGT GGGGAATCTGCCTTTGAGCGGGGGACAACATTTGGAAACGAATGCTAATACCGCATAAAAACTTTA AACACAAGTTTTAAGTTTGAAAGATGCAATTGCATCACTCAAAGATGATCCCGCGTTGTATTAGCTA GTTGGTGAGGTAAAGGCTCACCAAGGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACAT TGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGA AAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAG AAGAACGTTGGTGAGAGTGGAAAGCTCATCAAGTGACGGTAACTACCCAGAAAGGGACGGCTAAC TACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCG AGCGCAGGTGGTTTATTAAGTCTGGTGTAAAAGGCAGTGGCTCAACCATTGTATGCATTGGAAACT GGTAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATG GAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCCTGTAACTGACACTGAGGCTCGAAAGCGTGGGG AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGATGAGTGCTAGATGTAGGGAGCTAT AAGTTCTCTGTATCGCAGCTAACGCAATAAGCACTCCGCCTGGGGAGTACGACCGCAAGgTTGAAA CTCAAaGGAATTGACGGGGgCCCGCACAaGCGGTGGAGCATGTGGTTTAATTCGAAGCAaCGCGAGA aCCTTACCAGgTCTTGACATACTCGTGCTATtCCTAGAGATAGgAAGTTCCTTCGGGACACGGGATAC AGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA CCCCTATTGTTAGTTGCCATCATTAAGTTGGGCACTCTAACGAGACTGCCGGTGATAAACCGGAGGA AGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATG GTACAACGAGTCGCGAGACAGTGATGTTTAGCTAATCTCTTAAAACCATTCTCAGTTCGGATTGTAG GCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATAC GTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGGGAGTTGGGAGTACCCGAAGTAGGTTGCC TAACCGCAAGGAGGGCGCT	100	*Lactococcus lactis* subsp. *lactis* Vm214	1409

B1- B5: number of isolates, bp: base pairs

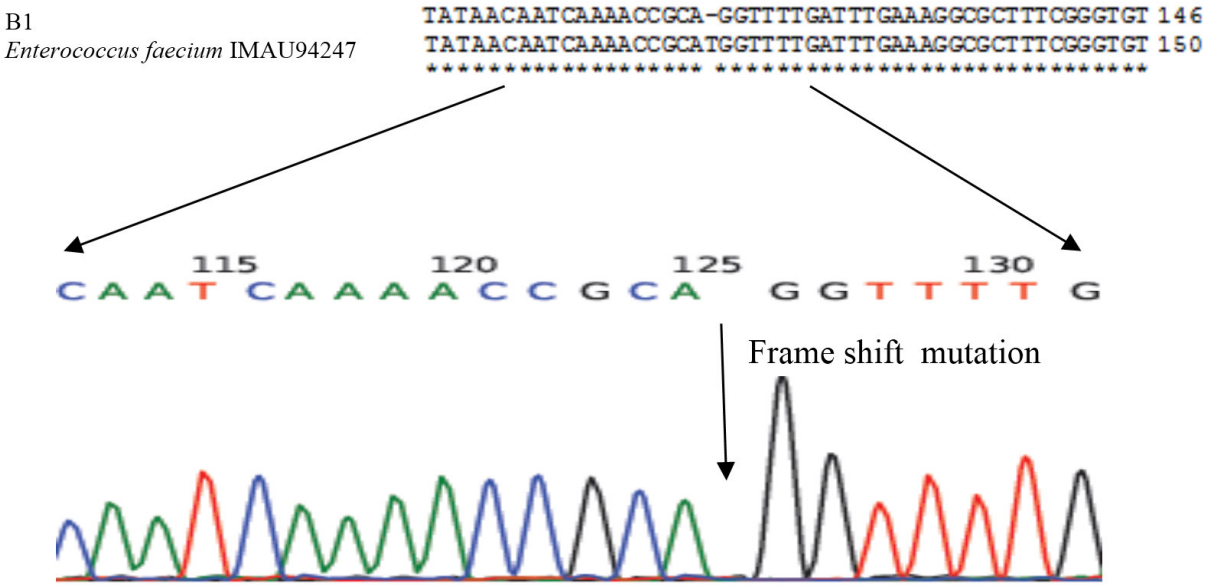

Figure (3. 5): CLUSTALW for comparison of nucleotide sequences alignment for 16SrDNA of B1 to strain *Enterococcus faecium* IMAU94247, showing frame shift mutation at the position 126bp.

4. Discussion

In our study isolates have appeared ovoid in pairs in B2 and B5, cocci in pairs or very short chains in B1and B3 isolates.

The two isolates B2 and B5 which were suggested as *Lactococcus lactis* (even later) have a close similarity in the most characteristics, they have agreed with (Tuber 2009) in morphological features as well as negative reaction for catalase, gas from glucose and acid from raffinose, with positive growth at 10°C, while differed from the same records by positive and variable growth in 6.5% NaCl and 45°C respectively, and from Ray (2005) in positive growth at pH 9.6. The positive growth in that pH was recorded in Wang *et al*. (2008) and Khay *et al*. (2011). The results have come as the same as Şahingil *et al*. (2011) in negative reactions of pyrrolidonylarylamidase (PYR), raffinose and arabinose and positive for mannitol.

Naphthyl-β-D-D-galactopyranoside or β-galactosidas (ONPG) and esculin were positive while hippurate negative in (Şahingil *et al*. 2011), whereas in (Wang *et al*. 2008) (ONPG) was negative. These results approached in somewhat from our isolates, which arranged as, positive, variable, negative for B2 and variable for three tests in respect to B5.

The positive and variable growth in 6.5% NaCl, 45°C and pH 9.6 for B2 and B5 may return to the locality of those isolates by which isolates could be affected by local Iraqi environment.

The characteristics, biochemical and physiological tests for B1 have been exclusively compatible with tests and characteristics of *Enterococcus faecium* in (Švec and Devriese, 2009) such as; growth positively at 10 and 45°C, 6.5% NaCl and pH 9.6; hydrolysis of esculin, pyrrolidonyl arylamidase (PYR), β- Galactosidase (ONPG); negative for catalase and gas from glucose (homofermentative), negative due to strain for hippurate, mannitol and raffinose.

The isolate B3 which at first has been suggested as *Enterococcus faecium* according to agreement with B1 and literature cited, changed later by sequencer to *Lactococcus lactis*.

So, positively growth at 45°C, 6.5% NaCl, pH 9.6, pyrrolidonyl arylamidase (PYR) and negative to mannitol may be explained by the locality of the strain in Iraq which might be affected by its environment.

4.1. Genetic identification by PCR and sequencing.

By applying PCR technique, we have observed that amplified region is found in 1500 bp approximately, which are agree with the result of (Hussein 2013) for all four isolates, because the 16S rDNA appears in this region in all bacteria.

Patel (2001) reported that for many years sequencing of the 16S rDNA gene has served as an important tool for determining phylogenetic relationships between bacteria and it is a powerful mechanism for identifying new pathogens in patients with suspected bacterial disease, and more recently this technology is being applied in the clinical laboratory for routine identification of bacterial isolates.

Throughout sequencing, we have obtained that the three isolates B2, B3 and B5 had identity of 100% with the reference strains *Lactococcus lactis* subsp. *lactis* TW35, *Lactococcus lactis* subsp. *lactis* Vm214 and

Lactococcus lactis subsp. *lactis* Vm214 respectively, in addition to the isolates B1 which had identity of 99% with the reference strain *Enterococcus faecium* IMAU94247, which led us to suggest the probability of obtained new strain.

By using of 16S rDNA sequence analysis, it had been demonstrated by Drancourt *et al*., (2000) that in the absence of an accepted cutoff value, they retained a 99% similarity as a suitable cutoff for identification at the species level and a 97% similarity as a suitable cutoff for identification at the genus level and while the introduction of these sharp values was necessary to analyze a large collection of unidentified isolates belonging to different genera, further evaluations need to be performed to assess the accuracy of these values, besides; because bacterial genera do not evolve at the same speed, it may be necessary to use different cutoff values depending on the bacterial genus under investigation.

In our study, we have assumed 1% difference (99% identity) with sequence of 16S rDNA from the reference strains in the identification of our suspected new strain. The different of the isolate B1has been expressed as a frame shift mutation by the deletion of nucleotide Thiamine which changed the encoded target amino acid. Chmagh (2013) and Hussein (2013) adopted 1% difference (99% identity) with sequence of 16S rDNA from the reference strains in the identification of their novel strains studied also. Both of point and frame shift mutations appeared in those identification studies.

We have concluded that raw animal milk is a rich source by a bacterial variety, in which, LAB is one of the dominant. Molecular identification such as sequencing may change the results of biochemical and physiological tests, so it must be as a complementary identification. In this study, a novel strain has been proposed.

Acknowledgement
All thanks to assist. Lecturer Khlood Abdulkareem/ College of Nursing, for providing chemicals and solutions of molecular study, in addition to manipulation of sequencing data.

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