



Molecular Identification and Phylogenetic Analysis of Lactic Acid Bacteria Isolated from Cow Raw Milk in Al-Kut -Iraq

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Received 2nd Aug 2023,
Accepted 19th Sep 2023,
Online 16th Oct 2023

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Abstract: The objective of “this study was to determine the genetic variability of lactic acid bacteria (LAB) present in locally sourced cow's milk. A total of 50 raw milk samples were collected from several local markets in Al-Kut. The specimens were cultured in the De man, Rogosa, and Sharpe (MRS) medium, which is particularly formulated to promote the growth of lactic acid bacteria. The presumed isolates were then identified by the use of polymerase chain reaction (PCR), which selectively targets the 16S rRNA gene, and afterwards followed by DNA sequencing”. The results revealed that lactic acid bacteria were present in 28% (14 isolates) of the samples, including two genera and five species. Specifically, there were 2 strains of *Lactococcus lactis* and 12 strains of *Lactobacillus* spp, including 5 *Lactobacillus Plantarum*, 4 *Lactobacillus gasseri*, 2 *Lactobacillus acidophilus*, and 1 *Lactobacillus delbrueckii*. The predominant Lactic acid Bacteria in the present study (*Lactobacillus Plantarum*) were furtherly tested for their antibacterial activity and the results showed that , *Lactobacillus Plantarum* exhibited potent antibacterial effects against indicator bacteria like “*Staphylococcus aureus* and *Staphylococcus epidermidis*, as well as pathogenic bacteria such as *E.coli* and *Pseudomonas*” *aeruginosa*.

The 16S rRNA gene sequencing and phylogenetic tree analysis indicated that all the obtained strains exhibited 99% homology with certain LAB strains published in the NCBI. Subsequently, the isolates were recorded in the NCBI.

Key words: lactic acid bacteria, Molecular characterization, cow milk, Al-Kut, Iraq.

Introduction

Lactic “Acid Bacteria (LAB) are essential for the manufacture and preservation of many fermented food products. The research mostly examined the advantages and impact on health of lactic acid bacteria in the current context of industrial food production (1,2). Lactobacilli are microorganisms that are non-spore-forming, Gram-positive rods” or coccobacilli. Lactobacilli are microorganisms that undergo fermentation, require a low level of oxygen, obtain energy from chemicals, and need nutrient-rich environments to thrive (3). Although certain strains may exhibit pseudocatalase activity, they are primarily catalase deficient. The analysis of the genome's DNA base composition revealed a GC concentration below 54% (4). Lactobacillus species are regarded as the primary and prevailing genus of LAB present in the gut of honey bees, as well as in humans and other animals' intestines (1,2). Lactobacillus spp. and Bifidobacterium spp., acting as probiotics, have the potential to significantly contribute to the enhancement of animal and human well-being (5,6). Prior studies have demonstrated that lactobacilli have a crucial role in preventing and treating a range of gastrointestinal illnesses in humans, including infectious enterocolitis, as well as enteric and colorectal malignancies (7-9).

Lactic acid bacteria (LAB) have a wide distribution in nature, being present in environments rich in carbohydrates, such as various types of food (including “dairy products, fermented meat, sour dough, vegetables, fruits, and beverages), as well as in the respiratory, gastrointestinal tract (GIT), and genital tracts of both humans and animals. They can also be found in sewage and plant material (10). Milk is often regarded as one of the most exceptional sources of LAB (lactic acid bacteria). The LAB (lactic acid bacteria) have been extracted from milk and fermented foods and have been granted the generally recognized as safe status (GRAS)”. They are extensively employed in the fields of food and medicine, owing to their probiotic characteristics (11). Various varieties of milk, such as goat, cow, and sheep, are globally manufactured for human use. Goat milk is less popularly consumed compared to cow milk and accounts for approximately 2% of the worldwide milk supply (12). The increased attention towards goat milk is mostly due to its superior iron bioavailability, elevated levels of fatty acids, and less allergenicity (13). Prior research has consistently found that the “microbiota in milk is predominantly made up of Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, and Streptococcus species. These bacteria are well-known for their probiotic and bacteriocinogenic activities (14-16). According to the NCBI scientific categorization database, the number of species belonging to the Lactobacillus spp. has risen to 172 in recent years. These significant discoveries are necessary to gather and examine the extensive sets of sequence data in order to unravel the evolutionary relationships among species of lactic acid bacteria. Furthermore, the growing accessibility of genomic data for lactic acid bacteria is a valuable opportunity to gain insights into the evolutionary lineage of these species (17). Our objectives were to evaluate the genetic diversity of the predominant lactic acid bacterium species found in the local raw” cow milk.

Martials and methods

Collection and Isolation of Bacteria from cow Raw Milk Samples

Fifty samples of local cow raw milk were collected from Al-kut local markets using sterile containers. To ensure the preservation of sample integrity, all collected samples were promptly transported to the laboratory in a cool box.

In the laboratory, one milliliter of each milk sample was aseptically transferred to 9 milliliters of De Man, Rogosa, and Sharpe broth (MRS broth). These samples were then incubated at 37°C for 24 hours. Following the incubation period, primary cultures were obtained, and all colonies present were subjected to purification through subculture on MRS agar. The purified colonies were incubated at 37°C for an additional 24 hours. Then, well-isolated, typical colonies representing potential lactobacilli based on “Bergey's manual of systematic bacteriology” were selected for preliminary screening [17].

“These tests were for catalase activity using H₂O₂, motility using stab method (0.4% agar in MRS broth) and gelatin liquefaction test using 12% gelatin in MRS broth”. Further confirmation of the bacterial identity was achieved through polymerase chain reaction (PCR) analysis and DNA sequencing.

Molecular detection of LAB species

Genomic DNA Extraction: The genomic DNA was extracted using the "Genomic DNA Extraction Kit (Geneaid, USA)" in accordance with the manufacturer's instructions.

PCR amplification of 16S rDNA gene:

PCR procedures were conducted to “amplify the 16S rDNA gene in LAB isolates. The 16S rDNA region was amplified using the universal primer set, consisting of 8F (5-AGAGTTTGTATCCTGGCTCAG-3) and 1492R (5-GGTTACCTTGTACGACTT-3)”, as specified in reference (1) and provided by Bioneer Company, Korea. The reaction consisted of 1 µl of both the “forward and reverse primers”, 3 µl of DNA template, and nuclease-free water was added to bring the final volume to 20 µl. The thermocycling protocols for this gene are provided in table (18). Subsequently, the PCR reaction mixture was vigorously mixed and subjected to centrifugation prior to its transfer to a biorad thermocycler (China). The PCR result was ultimately examined using a 1% (w/v) agarose gel.

Sequencing of PCR products

PCR product subjected to sequence analysis. A total of fourteen “PCR products of the specific gene were sent to MACROGEN/Korea “<http://dna.macrogen.com>” for gene sequencing. The raw sequences were visually inspected and modified using the Chromas software. The sequences were examined using the basic local alignment search tool (BLAST) to search for a comparable sequence in the national center for Biotechnology information database (NCBI) located at <https://blast.ncbi.nlm.nih.gov/>”. The phylogenetic tree was constructed utilizing the neighbor joining (NJ) technique and the MEGA11 software.

Assessment of bacteriocin production and effectiveness

The isolates “were cultivated in MRS broth at 37°C for 48 hours. Subsequently, the culture supernatant was collected by subjecting it to centrifugation at 12,000 xg for 10 minutes at 4°C. The agar diffusion assay, as described by (33) was used to determine the antibacterial activity. In this assay, 100 µL aliquots of the supernatants were placed in (6mm/d) wells on plates that were previously seeded with a 1% v/v solution of two gram-positive bacteria (Staphylococcus aureus and Staphylococcus epidermidis) as indicator strains. The antibacterial activity was then tested against two other pathogenic bacteria, E. coli and Pseudomonas aeruginosa. The plates were placed in an incubator at 37°C for 48 hours and growth inhibition zones” were recorded

Results and Discussion

The Structure and Morphology of Lactic Acid Bacteria.

Fifty samples of Cow milk were gathered from local markets in Al-Kut City. These samples were diluted and cultured on MRS selective medium, then incubated under anaerobic conditions at 37°C for 24-48 hours.

As illustrated in Figure 1 (A and B), single colonies exhibited precise boundaries and sleek surfaces; their diameters varied from 0.5 to 3 mm; and their centers were at least as tall as the margins. The colonies were colored white, translucent, or creamy yellow. When viewed through a microscope, the

cells exhibited a blue and purple staining pattern. They were spherical or rod-shaped and devoid of spores, which aligns with the descriptive features of lactic acid bacteria.

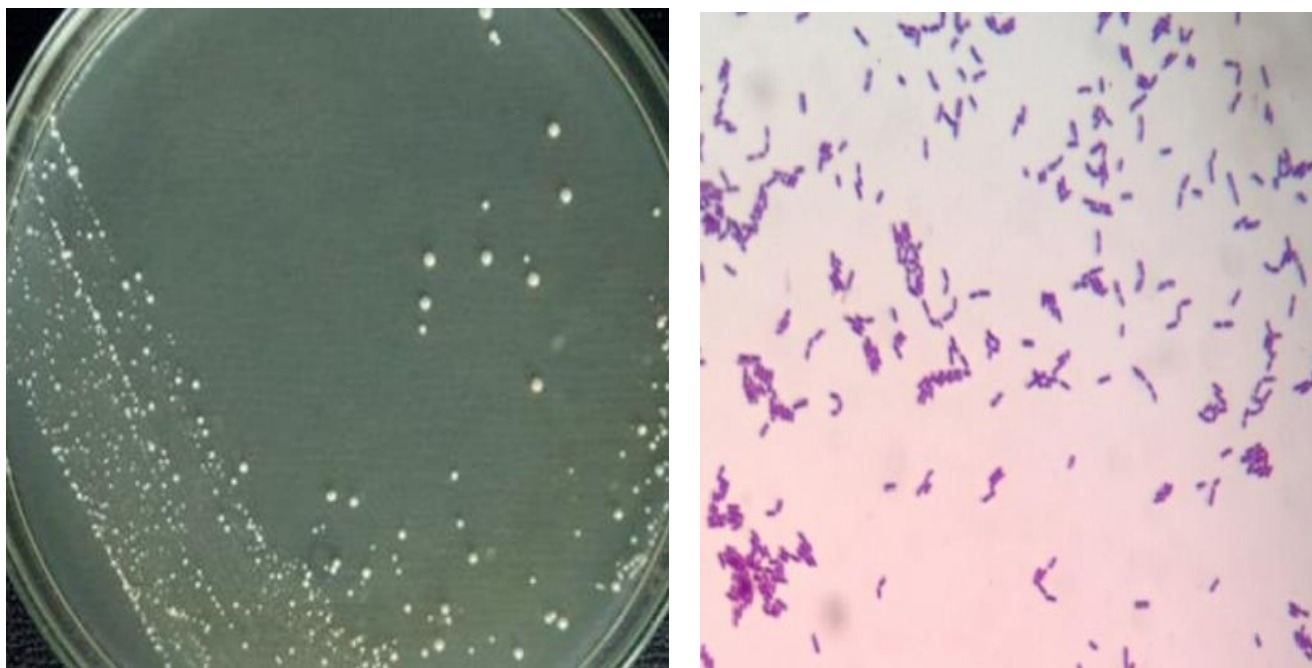


Figure 1: (A) Characteristics of Lactobacillus colonies after 48 hours of growth on MRS agar at 37°C . (B) Gram stains of Lactobacillus species .

In the present study, 14 isolates of lactic acid bacteria were isolated from cow's milk. Each isolate was cultured in anaerobic conditions at 37°C. For molecular identification, “only Gram-positive, non-motile, catalase-negative, and unable to liquefy gelatin microorganisms were considered.

For the 16S rRNA region, the size of the amplified PCR products was approximately 1500bp (figure 1). Comparisons of the 16S region sequences obtained from the isolates with those in GenBank allowed for the identification of the isolates at species levels . The retrieval results of GeneBank nucleic acid sequence database by adopting BLAST are as shown in table (1).

The evolutionary history was inferred using the NeighbourJoining method. Evolutionary analyses were conducted in MEGA11 figure (2) ”.

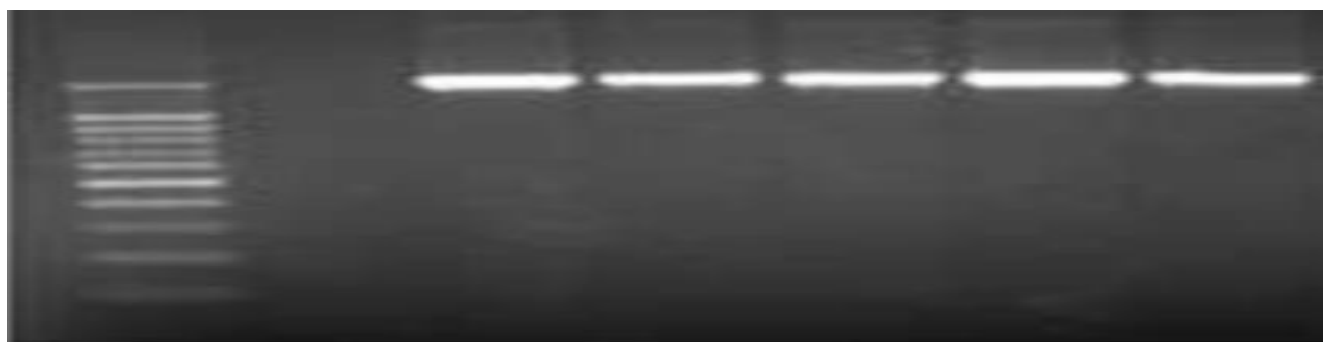


Figure 2. “Amlification of a 1500-bp 16S rRNA gene of LAB Bactria”.”

Table 1: The results of LAB isolate identification using 16S rRNA sequences

No	Identities	Identification results	Excisions numbers
1	99%	Lactobacillus Plantarum	➤ OR361765.1
2	99%	Lactobacillus Plantarum	OR361766.1
3	99%	Lactobacillus Plantarum	OR361767.1
4	99%	Lactobacillus Plantarum	OR361768.1
5	99%	Lactobacillus Plantarum	OR361769.1
6	99%	Lactococcus lactis	➤ OR361786.1
7	99%	Lactococcus lactis	➤ OR361787.1
8	99%	Lactobacillus acidophilus	OR361773 .1
9	99%	Lactobacillus acidophilus	OR361774 .1
10	99%	Lactobacillus gasseri	OR361781.1
11	99%	Lactobacillus gasseri	OR361782.1
12	99%	Lactobacillus gasseri	OR361783.1
13	99%	Lactobacillus gasseri	OR361784.1
14	99%	Lactobacillus delbrueckii	OR361789.1

As shown in Table 1, the sequences of the 16S rDNA region shared a 99% homology with those in the GenBank database among lactic acid bacteria strains. This homology satisfies the identification criteria, thus validating the identification. Table 2 provides a summary of the molecular biology identification results for fourteen strains of lactic acid bacteria.

Table 2: The “summary of the lactic acid bacteria based on 16S rDNA” sequencing

Strain name	Number	Proportion
Lactobacillus Plantarum	5	36%
Lactobacillus gasseri	4	29%
Lactobacillus acidophilus	2	14%
Lactococcus lactis	2	14%
Lactobacillus delbrueckii	1	7%

As “shown in Table 2, 14 strains of lactic acid bacteria belonged to 5 species of 2 genera”. Lactobacillus Plantarum were dominant in this study (36%) followed by Lactobacillus gasseri (29%), Lactobacillus acidophilus and Lactococcus lactis 14% for each one and Lactobacillus delbrueckii (7%).

Close results were obtained by (19,20,21) who found the predominance of Lactobacillus Plantarum and Lactobacillus acidophilus in the dairy products samples, however, they found different species and proportions of lactic acid bacteria in the dairy products.

The variation in the “genera of lactic acid bacteria present in dairy products manufactured in different regions” can be attributed to climates, geographical locations, and production conditions that encompass factors such as fermentation temperature, environment, inoculation amount, and time [22,23]. Additionally, distinct culture methods and mediums were utilized during production.

The primary “focus of this study was to examine the antibacterial properties of the dominant Lactic acid Bacteria in the present study, specifically Lactobacillus Plantarum. These bacteria were subjected to further testing to evaluate their ability to inhibit the growth of two indicator bacteria, Staphylococcus aureus and Staphylococcus epidermidis, as well as two pathogenic bacteria, Pseudomonas aeruginosa and E. coli”. These indicator organisms were isolated from various clinical samples. The findings of this investigation are presented in table 3.

Table 3: “Antimicrobial activity of isolated *Lactobacillus plantarum* against” pathogens.

Indicator bacteria	Antimicrobial activity	Degree of inhibition
<i>Staphylococcus aureus</i>	+++	Strong \approx 5mm
<i>Streptococcus epidermidis</i>	+++	Strong \approx 5mm
<i>E. coli</i>	-	No activity
<i>Pseudomonas aeruginosa</i>	++	Moderate \approx 3mm

Nevertheless, the findings presented in Table 2 were consistent with those reported by (25 , 26 , 27), Who demonstrated the “antibacterial efficacy of the bacteriocin derived from their isolates as an antibacterial agent”. The present study's isolates exhibited no activity against *E. coli*, which is known to produce bacteriocin on its own, as mentioned in reference (28).

Phylogenetic Analysis Results.

Fig. 3 illustrates that the fourteen strains of lactic acid bacteria that have been identified are classified into two clades, signifying the existence of two distinct genetic types. The 6th and 7th bacteria belong to the first clade with *Lactococcus lactis*. On the other hands , the remaining 12 bacteria that belong to the second clade with 4 species , *Lactobacillus Plantarum*, *Lactobacillus gasseri*, *Lactobacillus acidophilus* and *Lactobacillus delbrueckii*, suggesting that they are genetically related. Each species in this glade grouped together suggesting their genetic relatedness .however, *Lactobacillus delbrueckii* is more related to “*Lactobacillus acidophilus* than *Lactobacillus gasseri* and *Lactobacillus Plantarum*” . moreover , *Lactobacillus gasseri* is more “related to *Lactobacillus acidophilus* than and *Lactobacillus Plantarum*”. The 1th and 2th strains of *Lactobacillus Plantarum* exhibit a higher genetic similarity compared to other members of this species. Conversely, the eleventh strain of *Lactobacillus gasseri* shows a significantly greater genetic distance from other strains of the same species, indicating a significant level of genetic diversity.

The positioning of distinct strains in the phylogenetic trees aligns with the identification results, hence validating the high reliability of the 16S rRNA sequence identification method. Chen et al (29) state that lactic acid bacteria, like other bacteria, have the potential to acquire genetic material from the environment through horizontal gene transfer. This allows them to adapt and thrive in new environments. Thus, as “compared to traditional identification approaches, the 16S rRNA sequence analysis method” demonstrates greater accuracy and reliability, offering clear advantages in identifying lactic acid bacteria strains at the species level (30). Chen et al [29] illustrate that the lactic acid bacteria found in milk from various sources have undergone evolutionary changes to better suit their environment. In their investigation on the genetic variety of Lactic acid bacteria, Zhang et al. [31] discovered that the isolated strains exhibited a significant level of genetic diversity. Consequently, the genetic variety of lactic acid bacteria found in dairy milk is greatly influenced by natural selection and domestication, which is a result of the variations in geographical environments across different countries [32].

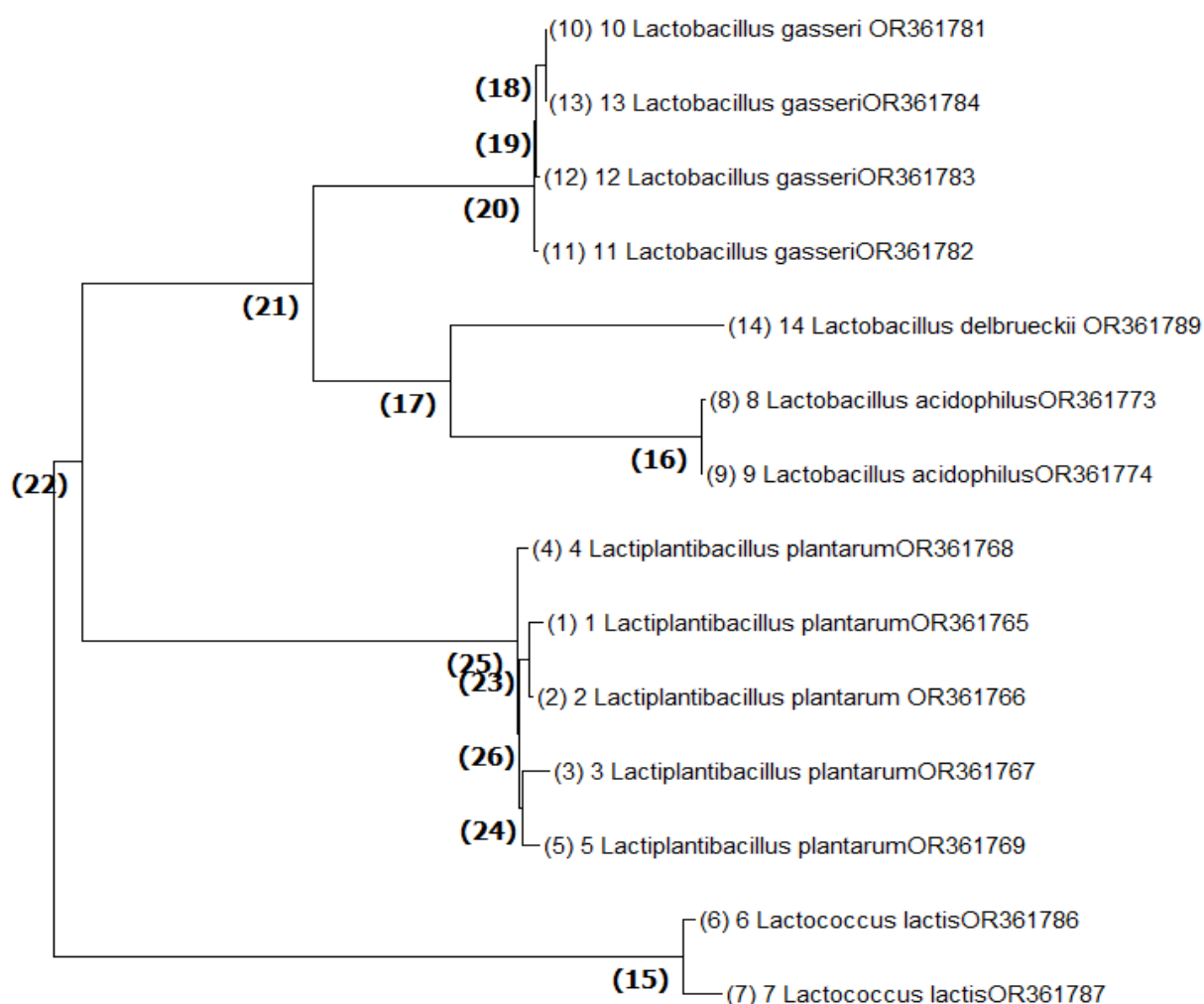


Figure 3. 16S rDNA-Based Phylogenetic Tree of 14 Lactic Acid Bacteria Strains .

Conclusions

It can be concluded, on the basis of the present findings, that various species of “beneficial lactic acid bacteria, which generate lactic acid and other metabolic products”, are present in cow milk. The 16S rDNA sequences of the Iraqi isolates exhibited 99% homology with lactic acid bacteria identified in the GenBank. Consequently, it is highly probable that the isolates originate from different countries.

Additional research should be conducted to better investigate the identification and characterisation of LAB at the strain level. Greater emphasis should be placed on the health and economic advantages of the selected LAB in order to enhance community well-being. Further experimentation is necessary to assess the potential use of these LAB as probiotics across various kinds of laboratory animals.

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