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Profiling of the Lactic Acid Bacteria Communities in Cow Milk based on 16S rRNA

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ABSTRACT

Background: This study aims to identify the types of lactic acid bacteria (LAB) in raw cow milk obtained from different locations in Babylon, Iraq using genetic analysis and study the phylogenetic relationship among them.

Methods: The study was based on 74 isolates collected from different milk samples. LAB was isolated on selective media from MRS agar after conducting a series of decimal dilutions. LAB was diagnosed depending on the morphological characteristics and nitrogenous base sequences for the *16S rRNA* gene, which was 1500 bp. the whole genome was extracted from all isolates.

Result: This study identified 74 isolated bacteria as gram-positive lactic acid bacteria (LAB), with most being cocci and the rest bacilli. Six LAB species were identified through genetic analysis (16S rRNA sequencing): *Lactobacillus rhamnosus, L. kefiri, L. curvatus, Lactococcus lactis, Pediococcus pentosaceus* and *Pediococcus acidilactici*. Phylogenetic analysis revealed relationships between these species and the study highlighted how environmental factors can lead to genetic variations within LAB. The research emphasizes the importance of molecular methods for identifying and understanding the genetic diversity of LAB in food and beverage samples.

Key words: 16S rRNA, Lactic acid bacteria (LAB), Milk samples, Phylogenetic tree.

INTRODUCTION

Lactic acid bacteria (LAB) have been classified as generally recognized as safe bacteria and have been extensively introduced into the food industry due to their technological and functional properties, such as the prevention of infections (Ale *et al.,* 2020). LAB are considered non-spore former, non-motile, tolerance acid, anaerobic bacteria but aerotolerant, cocci or bacilli, negative production for catalase, Gram-positive bacteria and fermenting carbohydrate to production the lactic acid at the end of metabolite Vinderola *et al.* (2019) and Sabatini (2010).

Meanwhile, they have been isolated from various dairy products including milk by many mediums like Man, Rogosa and Sharpe media (MRS) under anaerobic conditions (De Man *et al.,* 1960 and Ale *et al.,* 2020). LAB has possessed various biotechnological relevance, involving fermentation processes for food and feed and is capable of producing many important metabolites like organic acids, antimicrobial and aromatic compounds, exopolysaccharides, vitamins and bioactive peptides, which may improve the fermented food products through improving the sensory, nutritional and safety characteristics (Atanasova *et al.,* 2021). Additionally, LAB works to improve the food's nutritional value then the health of the human body (like having an immunomodulatory effect, restricting cancers and glucose serum levels and preventing infections) (Karem *et al.,* 2023; Muteab and AL-Abedy, 2025).

Mostly, the LAB requires specific techniques for diagnosing and isolating from environments. Therefore, *16S rRNA* gene sequencing was a suitable technique for the identification of species-level bacteria from various fermented foods and beverages (Delfederico *et al.* 2006). This technique used universal primers to amplify the *16S rRNA* gene followed by sequencing for amplified PCR products to detect and identify the LAB, so the molecular methodology was the most dependable approach to ascertain microbial diversity (Hugenholtz *et al.,* 1998; O'Sullivan, 2000; Al-Sharmani *et al.,* 2019; Habib *et al.,* 2020). The advantage of the phylogenetic tree is to analyze the evolutionary relationship among microorganisms depending on the variable regions for *16S rRNA* that consist of the various ratios of nitrogen base led to a deep

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taxonomy of different genera and species (Stackebrandt *et al.,* 1983; Neamah *et al.,* 2020; Nurye and Wolkero, 2023). The current study aimed to isolate and diagnose the natural LAB that existed in raw cow milk obtained from different locations in (Babylon, Iraq) using genetic analysis and studied the phylogenetic relationship among them.

MATERIALS AND METHODS

Sample collection and growth conditions

The study was conducted from February to October 2023 to isolate and identify the lactic acid bacteria (LAB) in raw cow milk. A seventy-four samples were collected and stored in an icebox until transported to the laboratory and started the isolation process.

LAB was isolated from samples by adding 11 ml milk samples to 99 ml (0.1) Peptone water serially diluting to 10^{-3}. About (0.1) ml of each appropriate dilution was plated into MRS (Man, Rogosa and Sharpe) agar and incubated at 37°C for 3 days under an aerobic condition. After incubation, individual LAB colonies were randomly picked up and purified by reculturing on MRS agar. The isolated LABs were purified according to their cultural features (colored by gram stain, characteristics microscopically (cocci or bacilli) described by Leboffe and Pierce (2011), Macfaddin, (2000). Finally, the pure plate cultured of each isolate was stored at 7°C for the molecular analysis.

Isolation of bacterial genomic DNA

According to the manufacturer's instructions, the genomic DNA of LAB was extracted using a Genomic DNA Mini Kit from Zymo (Zymo Research Company, USA).

PCR amplification of 16S rRNA region

PCR amplification was done to confirm the identity of the bacterial strain using 16S rRNA gene amplified from the genomic DNA with 27F (5′AGAGTTTGATCMTGGCTCAG3′) and 1492R (5′CTACGGCTACCTTGT TACGA3′) universal primers to get an amplicon size of 1500 bp as described by Lagacé *et al.* (2004). Amplification was performed in 50 µl reaction volume with 10 × buffer, 2 mM dNTPs, 3 U/µl Taq DNA polymerase, 0.33 µl; 100 ng/µl of each primer, 2 µl template DNA, 1µl and H_2O 34.67 µl in an ASTEC thermocycler (Gene Atlas, Japan). PCR conditions 95°C for 2 min (denaturation), 45°C for 1 min (annealing) and 72°C for 1 min (extension). The ultimate extension was 72°C for 10 min after 40 cycles. Following approach, Amplicons were subjected to electrophoresis and visualization on 0.8% agarose gel using a 100 bp ladder as size markers (Green and Sambrook, 2012; Kamaluddin *et al.,* 2021; Al-Salami *et al.,* 2019; Aljuaifari *et al.,* 2019).

DNA sequencing and alignment

PCR-amplified products were sequenced for both directions forward and reverse primers. The obtained nucleotide sequences were aligned and compared with those of the strains in the NCBI database using the Basic Local Alignment Search Tool BLASTN (Thompson *et al.,* 1994; AL-Abedy *et al.,* 2019; Mahdi *et al.,* 2020). The phylogenetic tree was performed and based on the neighbor-joining method by the MEGA6 software (Saitou and Nei, 1987; Tamura *et al.,* 2013; Odeh *et al.,* 2021).

RESULTS AND DISCUSSION

Isolation and purification

About forty-seven LAB were isolated from fifteen raw cow milk samples collected from different areas in Babylon City, Iraq. These bacterial genera were initially purified based on the morphological and microscopically characteristics (cocci or bacilli depending on gram stain). Results showed that all the isolated bacteria were gram-positive, microscope examination revealed that most of the LAB including 52 isolates were found cocci, whereas the 22 LAB isolates were found to be bacilli. As a result, the LAB isolates were classified into five grouped genera that were: (29.85%) *Lactobacillus*, (27.99%) *Lactococcus*, (11.36%) *Pediococcus* and the rest distributed among *Leuconostocs and Enterococcus*.

In some previous studies conducted by El-Ghany *et al.* (2016) and Allaith *et al.* (2022) found that LABs have cocci or bacilli shapes, are homo-fermentative or hetero-fermentative and have positive or negative gram stain.

In the current study, *Lactobacillus* was the prevailing genus among the other genera isolated from the collected raw cow milk samples with a ratio (of 29.85%). These results agreed with Badis *et al.,* (2004) who indicated that the *Lactobacillus* species were the most dominant species isolated from raw goat's milk involving *L. plantarum, L. helveticus, L. brevis* and *L. delbrueckii subsp. Bulgaricus.*

Lactobacillus species were isolated and identified from traditional fermented milk by El-Ghany *et al.* (2016) and Allaith *et al.* (2022). Species of Lactobacillus, Leuconostoc and Lactococcus were identified as prevailing isolates in Dhan traditional butter by Guetouache *et al.* (2014). Holzapfel (1998) and Osmanagaoglu (2010) reported that the LAB species within the *Pediococcus*, *Lactobacillus* and *Lactococcus* are commonly utilized as probiotics for animals and humans and to produce and preserve various foods.

In Egypt, El-Ghany *et al.* (2016) and Allaith *et al.* (2022) lactobacillus species were isolated and identified from traditional fermented milk. As shown by Guetouache *et al.* (2014) species of *Lactobacillus, Leuconostoc* and *Lactococcus* were identified as prevailing isolates in Dhan traditional Butter. Holzapfel, (1998) and Osmanagaoglu, (2010) reported that the LAB species within the *Pediococcus, Lactobacillus* and *Lactococcus* are commonly utilized as probiotics for animals and humans and to produce and preserve various foods.

Molecular identification of LAB isolates

Seventy-four PCR amplified of *16S rRNA* products for the LAB isolates with a band size of approximately 1500 bp compared with the 1500 bp for the marker as displayed in (Fig 1). In this regard, (Stackebrandt *et al.,* 1983;

El-Ghany *et al.,* 2016; Allaith, 2018; Allaith *et al.,* 2022; AL-Shammari *et al.,* 2023) reported the significant role of the *16S rRNA* gene in diagnosing the LAB species because the *16S rRNA* gene is considered a universal and conserved region.

Sequencing of PCR products amplified and alignment with NCBI

PCR product amplified from each LAB isolate (Fig 1) sequenced in two directions to identify the isolated bacteria at the species level. The nucleotide sequence obtained from each bacterial isolate was analyzed using a GenBank BLASTN alignment (El-Ghany *et al.,* 2016; Allaith *et al.,* 2022). Results showed that all the identified isolates belong to LAB distributed among the species *Lactobacillus rhamnosus* (12%), *Lactobacillus kefiri* (8%), *Lactobacillus curvatus* (8%), *Lactococcus lactis* (33%), *Pediococcus pentosaceus* (2%) and *Pediococcus acidilactici* (8%).

The similarity % among local isolates and various strains in NCBI

The aligned nucleotide sequences were obtained from the bacterial isolates with the same strain in NCBI that reached complete similarity (100%) (El-Ghany *et al.,* 2016). The nucleotide sequences of the *L. rhamnosus* isolates in this study were analyzed and compared with those *L. rhamnosus* isolates previously registered in NCBI (Fig 2). The genetic similarity percentage of nucleotide sequences ranged between 99-100%. It was the closest to many isolates previously recorded such as those identified in China (CP053619 and CP019305), South Korea (MT32 2928) and Thailand (MN435970) with a nucleotide sequence similarity of 100%; whereas the lowest sequence homology (99%)was noticed with some *L. rhamnosus* isolates previously identified in many countries in the world such those identified in South Korea (MN533908 and USA (HQ811818). Similarly, El-Ghany *et al.* (2016) found in their study that *L. rhamnosus* strain was used as a control to compare with the rest of the LAB isolates that were identified by *16S rRNA* and alignment with NCBI.

L. curvatus isolates showed the highest similarity in the nucleotide sequence, with a match of 100% to the *L. curvatus* LC129555 strain in China (Fig 3). Whereas the *L. curvatus* isolate had the lowest similarity percentage with the *L. curvatus* strains identified in many countries, such as South Korea (MN372055.1), Peru (MG031212) and China (EU855223). Additionally, the highest nucleotide sequence similarity reaching 100% was found with the *L. kefiri* isolates in Turkey (MH549129) and Italy (NR_042 230) while the lowest similarity nucleotide sequence of the *L. kefiri* isolate was identified in China (HM218366) reaching 99% (Fig 4). These results agreed with Guetouache *et al.* (2014) that the species of *Lactobacillus*, *Leuconostoc* and *Lactococcus* were identified as prevailing isolates in fermented food by 16S rRNA and alignment with NCBI.

Additionally, *P. acidilactici* isolates had the highest similarity of the nucleotide sequence with a match of 100% *P. acidilactici* AF515229 strain in Japan (Fig 5). Whereas the *P. acidilactici* isolate had the lowest similarity percentage with the *L. curvatus* CP006854 strains identified in Denmark reaching 98%. The results displayed as shown in (Fig 6) comparison of the nucleotide sequence obtained from *P. pentosaceus*, identified in this study, showed that there is a 100% similarity with most of the *P. pentosaceus*

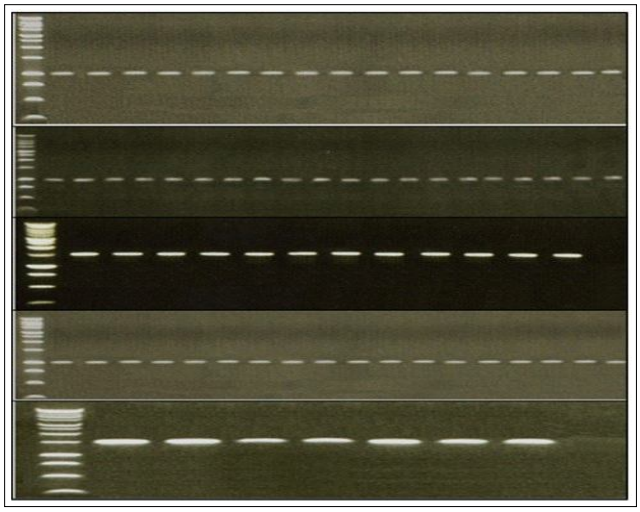
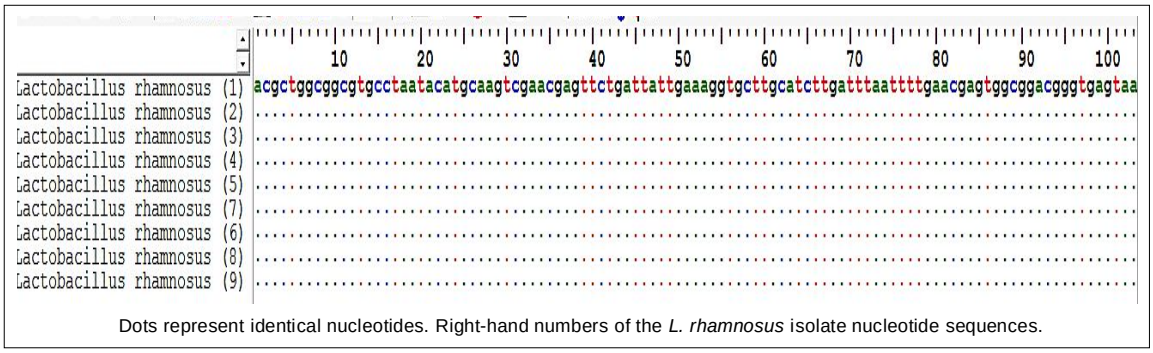


Fig 1: PCR products amplified from 74 LAB isolates obtained from milk samples by agarose gel electrophoresis.

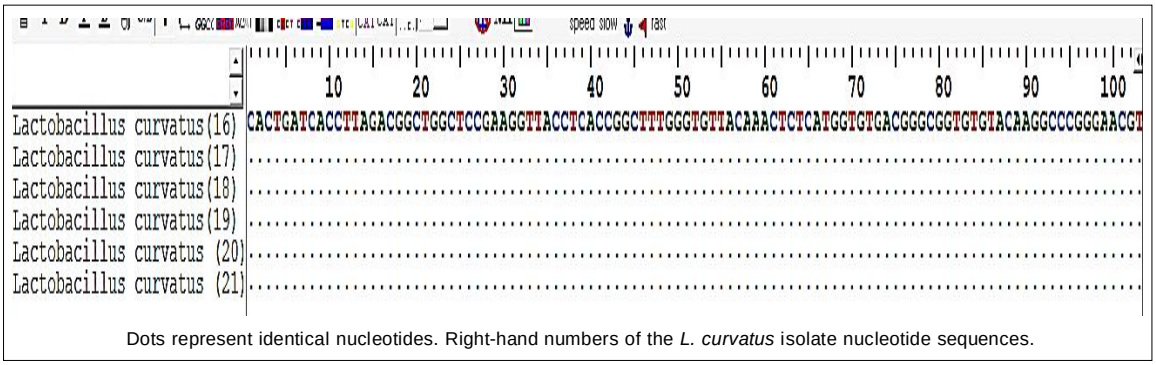


Dots represent identical nucleotides. Right-hand numbers of the *L. rhamnosus* isolate nucleotide sequences.

Fig 2: *L. rhamnosus* isolates 1, 2, 3, 4, 5, 6, 7, 8 and 9 had identical nucleotides sequences.

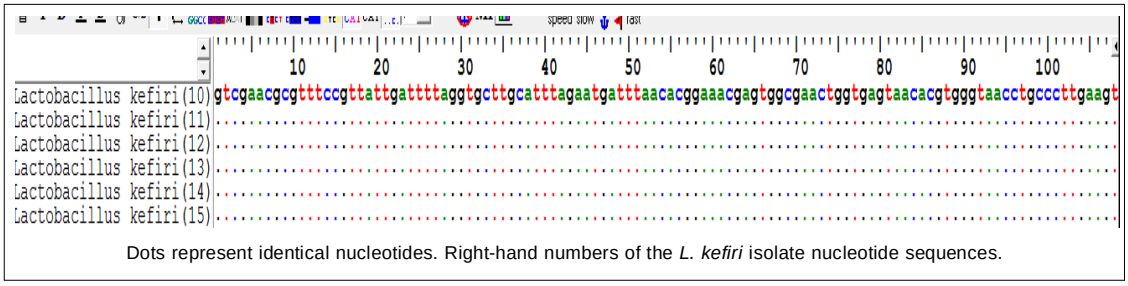
isolates, deposited in NCBI, such as the *P. pentosaceus* MH045191 in India, (MT510516, MT516146 and MT464432) in China and (CP028269) in Korea. In this regard, El-Ghany *et al.* (2016) reported that *P. parvulus* F1030 was isolated from fermented food and identified by 16SrRNA sequencing by alignment with NCBI. Similarly, Immerstrand *et al.* (2010) found in their study that isolates and diagnoses the *P. parvulus* 2.6 by 16S DNA sequencing and alignment with NCBI.

Morphological and molecular identification results also demonstrated that 25 out of 74 isolates obtained in this study belong to the species *L. lactis*. The alignment analysis of the sequence for *L. lactis* had 100% similarity with most of *L. lactis* strains recorded previously in NCBI such as *L. lactis* (MN749817) in Korea, (MN749817 and MH549138) in Morocco, (HE805077) in Thailand, (MH549 123 and MH549135) in Turkey and (NR-040955) in the USA. The other isolates available in NCBI gave a 99%



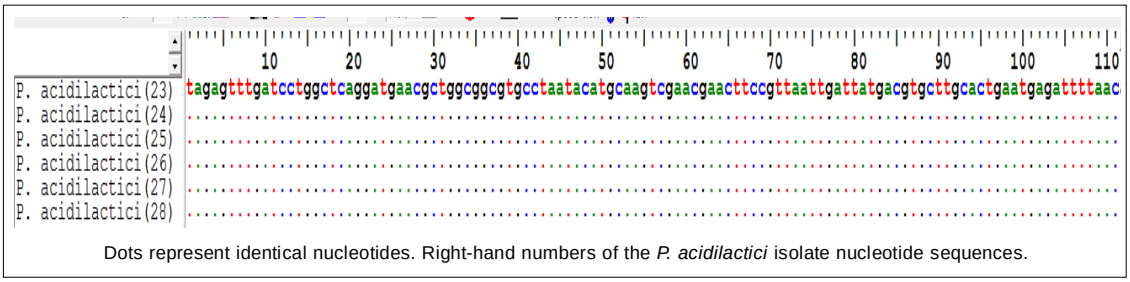
Dots represent identical nucleotides. Right-hand numbers of the *L. curvatus* isolate nucleotide sequences.

Fig 3: *L. curvatus* isolates 16, 17, 18, 19, 20 and 21 had identical nucleotides sequences.



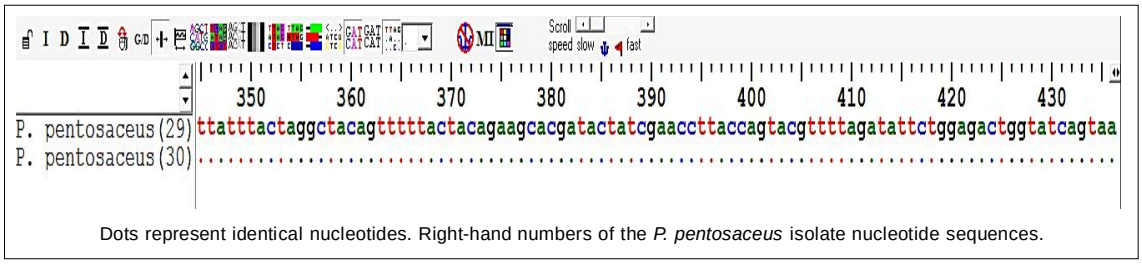
Dots represent identical nucleotides. Right-hand numbers of the *L. kefiri* isolate nucleotide sequences.

Fig 4: *L. kefiri* isolates 10, 11, 12, 13, 14 and 15 had identical nucleotide sequences.



Dots represent identical nucleotides. Right-hand numbers of the *P. acidilactici* isolate nucleotide sequences.

Fig 5: *P. acidilactici* isolates 23, 24, 25, 26, 27 and 28 had identical nucleotide sequences.



Dots represent identical nucleotides. Right-hand numbers of the *P. pentosaceus* isolate nucleotide sequences.

Fig 6: *P. pentosaceus* isolates 29 and 30 had identical nucleotide sequences.

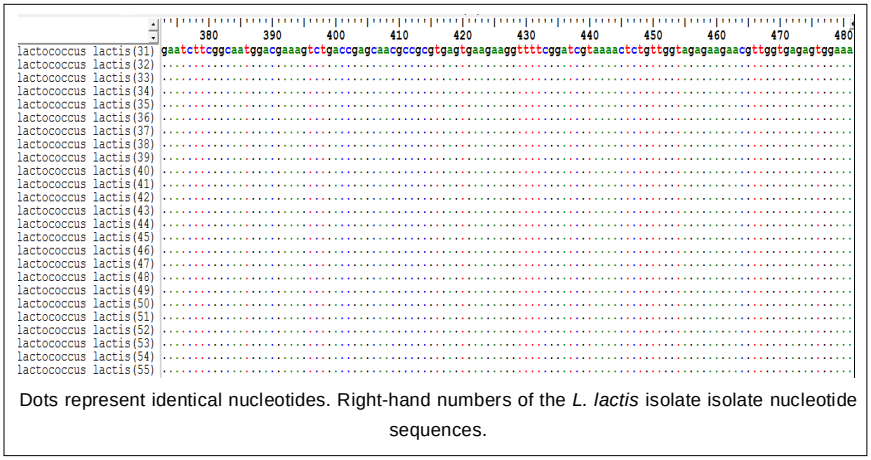
similarity with *L. lactis* strains in NCBI such as (NR_116 443) in the USA and (AM406671) in Ireland as shown in (Fig 7). These results agreed with Nomura *et al.,* (2006) that the *L. lactis* were isolated from milk and plant and identified by *16S rRNA* and alignment with NCBI, subsequently studying the genetic and phenotypic traits for it.

The phylogenetic tree analysis

An unrooted phylogenetic tree was generated using the Neighbor-Joining method based on comparing the nucleotide sequences generated from (LAB) isolates including (*L. curvatus, L. kefiri, L. rhamnosus*) and *L. plantarum* (as a control) as shown in (Fig 8) (Saitou and Nei, 1987). The most accurate tree had a total branch length with evolution distances estimated following the maximum likelihood approach (Tamura *et al.,* 2013) indicating the number of base changes per site. The results of the phylogenetic analysis proved the tree had two clades distributed among 22 isolates, the first clade consisted of Polyphyletic groups that had various species that lacked a common ancestor, including (1,2,3,4,5,6,7,8,9 isolates) belonging to *L. rhamnosus*, (10,11,12,13,14,15 isolates) belonging to *L. kefiri* and (22) isolate belonging to *L. plantarum*. Besides, the second clade consisted of monophyletic groups including (16, 17, 18, 19, 20 and 21) isolates belonging to *L. curvatus*.

On the other hand, an unrooted A phylogenetic tree was generated using the Neighbor-Joining method based on a comparison of the nucleotide sequences generated from the isolates *P. acidilactici* and *P. pentosaceus* as shown in (Fig 9) (Saitou and Nei, 1987).

The most accurate tree had a total branch length with evolution distances estimated following the maximum likelihood approach (Tamura *et al.,* 2013), indicating the number of base changes per site. The results of the phylogenetic analysis proved the tree had two clades of



Dots represent identical nucleotides. Right-hand numbers of the *L. lactis* isolate isolate nucleotide sequences.

Fig 7: *L. lactis* isolates numbered 31-55 had identical nucleotide sequences.

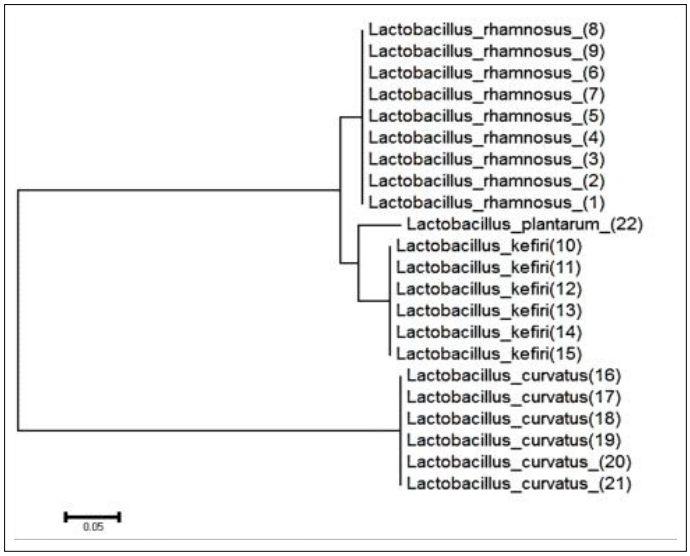


Fig 8: The Neighbor-Joining method was used to create an unrooted phylogenetic tree from lactic acid bacteria (LAB) nucleotide sequences (*L. curvatus*, *L. kefiri* and *L. rhamnosus)*.

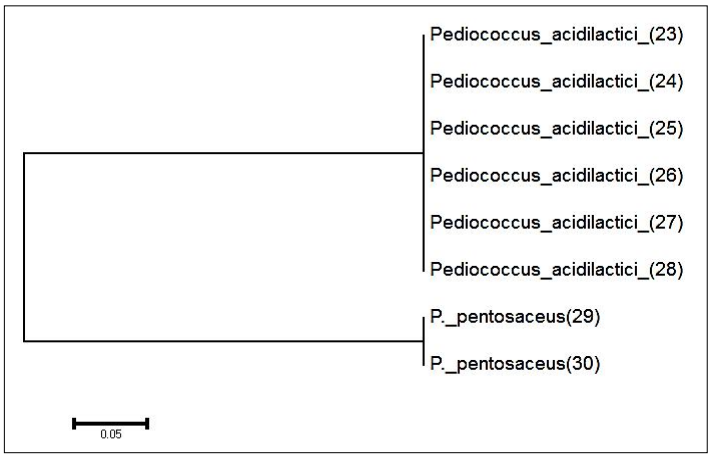


Fig 9: A Neighbor-Joining phylogenetic tree was created by comparing the nucleotide sequences of *P. acidilactici* and *P. pentosaceus*.

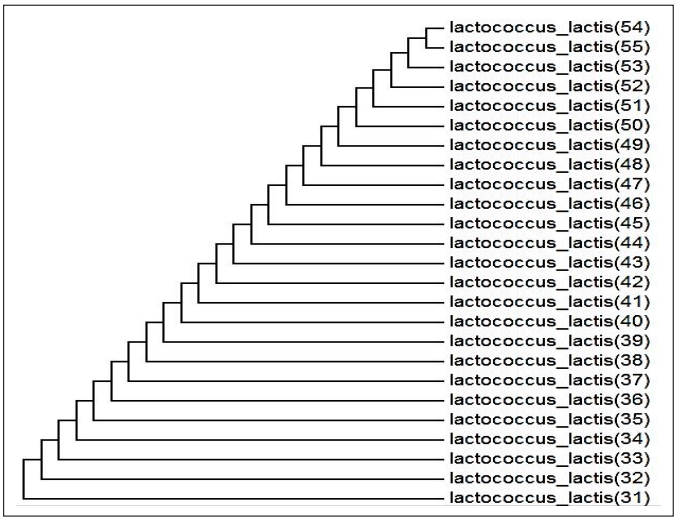


Fig 10: A Neighbor-Joining phylogenetic tree was created by comparing the nucleotide sequences of *L. lactis* isolates.

monophyletic groups (lacked a common ancestor) distributed among 8 isolates including (23, 24, 25, 26, 27 and 28 isolates) belonging to *P. acidilactici* and 29, 30 isolates belonging to *P. pentosaceus*.

Moreover, an unrooted phylogenetic tree was generated using the Neighbor-Joining method based on comparing the nucleotide sequences generated from *L. lactis* isolates (Fig 10) (Saitou and Nei, 1987; Alhissnawi *et al.,* 2024). The most accurate tree had a total branch length with evolution distances estimated following the maximum likelihood approach (Tamura *et al.,* 2013), indicating the number of base changes per site. The results of the phylogenetic analysis proved the tree had two clades of paraphyletic groups (lacked a common ancestor) distributed among 24 isolates including (31-55 isolates) belonging to *L. lactis*.

The advantages of studying the phylogenetic tree relationship among LAB species are in their interrelationships. Furthermore, environmental factors affected bacterial evolution that led to the detection of polymorphism in LAB because of the variation sequencing of nucleotides in the *16S rRNA* gene (Romanenko *et al.,* 2008; Jasim and Maaroof, 2017; Mnati *et al.,* 2021; Al-Shugeairy *et al.,* 2021).

CONCLUSION

The current study aimed to isolate and diagnose the natural LAB that exists in raw cow milk obtained from different locations in (Babylon- Iraq) Babylon, Iraq, using genetic analysis (16S rRNA gene) and a study examining the phylogenetic relationships among them. The isolation and diagnosis of the natural LAB Identified about 74 lactic acid bacteria distributed within the genera *Lactobacillus, Lactococcus, Pediococcus* and others were identified. The research emphasizes the importance of molecular methods for identifying and understanding the genetic diversity of LAB in food and beverage samples.

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Disclaimers

The views and opinions expressed in this article are solely those of the authors and do not necessarily reflect the official policies or positions of their affiliated institutions.

Informed consent

Not applicable. This study did not involve human or animal subjects. The research was conducted exclusively on microorganisms isolated from milk samples under controlled laboratory conditions.

Conflict of interest

The authors declare no conflict of interest regarding the research, authorship or publication of this article.

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