Molecular identification and phylogenetic analysis of lactic acid bacteria isolated from goat raw milk

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(Received July 08, 2019; Accepted August 27, 2019; Available online May 21, 2020)

Abstract

The aim of this study was to identify the genetic diversity of lactic acid bacteria (LAB) isolated from the local goat's milk. A total of 100 raw milk samples were collected from the different Basrah local markets. All the samples were cultured in the De man, Rogosa, and Sharpe (MRS) medium which enhances the growth of lactic acid bacteria. The result of the study showed that the only 64 lactic acid bacteria isolated gave the Gram-positive and catalase-negative were 64 (64%). All the suspected isolates were detected and identified by using polymerase chain reaction (PCR) targeting the 16S rRNA gene and DNA sequencing. The sequencing results showed that 9 strains belong to *Lactococcus* spp. and 6 strains belong to *Lactobacillus* spp. and all tested isolates had similarity over 99% with those recorded in the GenBank of The National Centre for Biotechnology.

Keywords: Lactic acid bacteria, Goat, Milk, Phylogenetic analysis

التشخيص الجزيئي والتحليل النسلي لبكتريا حامض اللاكتيك المعزولة من حليب الماعز الخام في العراق

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الخلاصة

هدفت هذه الدراسة الى دراسة التباين الوراثي لجراثيم حامض اللاكتيك والمعزولة من حليب الماعز. حيث تم جمع ١٠٠ عينه من الحليب من مختلف اسواق البصرة وتم زرع هذه العينات على الوسط المحفز لنمو هذه الجراثيم. بينت النتائج عن وجود ٦٤ عزله من هذه الجراثيم والتي اعطت ايجابية لصبغة غرام وسلبية لانزيم الكاتالايز بنسبة ٦٤%. لقد تم تشخيص جميع العزلات باستخدام تفاعل تسلسل البلمرة لجين ١٦ اس الرايبوزي. وبينت النتائج عزل ٩ سلالات تعود الى اللاكتوكوكس و ٦ سلالات تعود الى اللاكتوباسيلس وكانت جميع السلالات المختبرة لتتابع القواعد النتروجينية تشابه بنسبة ٩٩% مع تلك المسجلة في بنك الجينات العالمي.

Introduction

Over several centuries, Lactic Acid Bacteria (LAB) have an essential role in the manufacture and preservation of many fermented food products. The most important lactic acid bacterial genera namely *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Wissella1*. Many of the studies were focused on benefits and health of the lactic acid bacteria in the present industrial food manufacturing (1,2). Lactobacilli are Gram-positive rods or coccobacilli, non-spore-forming microorganisms. Lactobacilli is a fermentative, microaerophilic, chemo-organotrophic, and requiring rich

media to grow (3). They are a catalase negative, even if the pseudocatalase activity can sometimes be present in some strains. The DNA base composition of the genome, showed that the GC content is lower than 54% (4). *Lactobacillus* species are considered as the most important and also dominant genus of LAB found commercially in honey bee gut, also human, and intestines of other animals (1,2). As probiotics, *Lactobacillus* spp. and *Bifidobacterium* spp. could play an important role in the promotion of animal and human health (5,6). Many of the previous investigations showed that the lactobacilli have significant effects on the prevention and treatment of various human gastrointestinal disorders, infectious enterocolitis, besides enteric and colorectal cancers (7-9). In domestic ruminants, these bacteria play an important role in improving nutritional efficiency (10-12). The LAB are widely distributed in nature, they are found in the environments where carbohydrates are available, such as food (dairy products, fermented meat, sour dough, vegetables, fruits, and beverages), respiratory, gastrointestinal tract (GIT), genital tracts of humans and animals, in sewage, and the plant material (13). Milk considered as one of the best sources for the LAB. The LAB has been isolated from milk and the fermented foods and also have been conferred as generally recognized as safe status (GRAS) and has widely been used in food and medicine, due to their probiotic attributes (14). Different types of milk, including goat, cow, and sheep, are produced worldwide for human consumption. Goat milk is consumed less than cow milk and represents about ~2 % of the global milk source (15). Goat milk has gained interest mainly because of its iron bioavailability, the higher concentration of fatty acids and the lower allergenicity (16).

Many of previous studies had been reported that the microbiota in goat milk is composed primarily of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, and *Streptococcus* species, bacteria with known probiotic and bacteriocinogenic properties (16-18). In recent years, according to NCBI scientific classification database, the members of the *Lactobacillus* spp. increased up to 172 species. These huge findings are needed to collect and analyze the larger sequence data sets in order to disentangle the phylogenetic relationships among lactic acid bacteria species. Moreover, the increasing availability of the lactic acid bacteria genome sequence data offers a good opportunity to understand the evolutionary history of the lactic acid bacteria species (19). To date, investigation of microbes in milk in the studied area were applied for *Brucella* (20,21); *E. coli* (22-24); *Staphylococcus aureus* (25-27); *Listeria monocytogenes* (28); *Bacillus cereus* (29,30). None of these researches investigated LAB (31). Therefore, our objectives were aimed to characterize the genetic diversity of the main lactic acid bacterial species that contained in the local raw goat milk.

Materials and methods

Samples collection and bacterial isolation

One hundred sample of local goat raw milk were collected from Basrah local markets by using sterile container. All the samples were directly transferred to the laboratory by cool box. Then, one ml of milk samples was added to 9 ml of De Man, Rogosa and Sharpe broth (MRS broth) and incubated for 24 h at 37°C. All the colonies from primary cultures were purified by subculture on MRS agar and then incubated at 37°C for 24 hr. The suspected colonies on MRS agar were also identified by the Gram staining and biochemical tests. Further confirmation was done by polymerase chain reaction (PCR) and analysis of DNA sequencing.

Molecular detection of LAB species

DNA extraction

The total genomic DNA was extracted using AccuPrep® Genomic DNA Extraction kit according to the manufacturer's instructions (Bioneer/Korea). The 16S rRNA gene primers used for amplification and detection of *Lactobacillus* spp, the oligonucleotide for universal 27F 5-AGAGTTTGATCCTGGCTCAG-3', and for Universal 1392R 5-GGTTACCTTGTTACGACTT-3' at product size 1350 bp. The PCR reaction was performed in 20 µl reaction mixtures with 1 µl (10 pmol/µl) for forward primer and reverse primer, 5 µl ready to use master mix (Bioneer/Korea) and 8 µl nuclease-free water. Finally, the 5 µl DNA template was added to each reaction tube. For genes amplification, the PCR program was used: an initial denaturation step (94°C for 3 min), the second step is consist of denaturation, annealing, and extension (35 cycles at 94°C for 45 sec, and 56°C for 45 min, 72°C for 45 sec), and final extension is 72°C for 7 min.

The PCR products were subjected to electrophoresis in 1.5% agarose gel (32). A 100-bp DNA ladder (KAPA Universal DNA Ladder (cat # KK6302)) was used as a molecular weight marker and the gels were stained with red safety DNA staining, examined and photographed under UV transilluminator (Vilber-Lourmater UV light EEC /France). Fragment sizes of approximately 1350 bp were verified as a positive for the universal 16S rRNA gene.

Sequencing of PCR products

Twenty-one PCR products of targeted gene were sent to the MACROGEN/Korea "http://dna.macrogen.com" to get the gene sequencing. The raw sequences were visually reviewed and edited by using the Chromas software. The sequences analysis by using basic local alignment search tool (BLAST) to search for a similar sequence in the national center for Biotechnology information database (CNBI) https://blast.ncbi.nlm.nih.gov/. The phylogenetic

tree was constructed by using the neighbor joining (NJ) method, by MEGA10 software.

Results

Isolation of lactic acid bacteria

The total number of lactic acid bacteria isolated from goat's milk were 64 isolates. All isolates were grown at 37°C under anaerobic conditions. The isolates were Gram positive, non-motile, and catalase negative (Table 1). The PCR technique was done for these strains (Figure 1). Averagely, 1350 bp was obtained per sequence, which compared with those in GenBank by using the BLAST program (http://www.ncbi.nlm.nih). On one hand, the result of this study appeared that the 15 isolates diagnosed the lactic acid bacteria (Table 2). On the other hand, the phylogenetic relationship of the trial sequence and its close relations were investigated by using MEGA 10 software the phylogenetic tree was constructed and the same software was also used for reduced all positions containing gaps and missing data in the trail sequence (Figure 2 and 3).

Table 1: The number and percentage of lactic acid bacteria recovered from 100 milk samples

Number of positive	No.	%
No. of MRS culture Positive	64	64%
No. of PCR Positive	15	15%
No. sequence Positive	15	15%

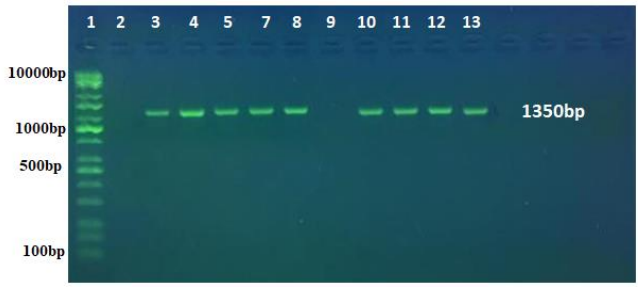

Figure 1: PCR product the band 1350bp. The product was electrophoresis on 1.5% agarose. Lane 1: DNA ladder (100bp), Lanes 3-8 and 10 -13 positive PCR results, Lanes 2 and 9 negative PCR.

Discussion

The present study showed that the percentage of suspected lactic acid bacteria isolated from goat milk was 64% (64/100). The percentage of frequency of lactic acid bacteria isolates based on PCR and sequence analysis was 15%. In this study the MRS medium was used for primary identification of the genus *lactobacillus* and other lactic acid bacteria because of the constrain growth of other species in this medium (33).

Additionally, Lopez-Diaz *et al*. (34) also improved the same results of the genus *Lactobacillus* was predominant in MRS. In our study, the percentage rate of the lactic acid bacteria isolated was 23.4% (15/64), this finding can be attributed to the presence of some cocci-shaped lactic acid bacteria in this medium can be explained by the low selectivity of this medium that allows the growth of other lactic acid bacteria genera (35).

Table 2: The identification results of 15 isolates of LAB by 16S rRNA sequences

Sample number - Source	Identities	GenBank ID
1. *Lactococcus raffinolactis*	99%	KC951911.1
2. *Lactococcus raffinolactis*	99%	KC951911.1
3. *Lactococcus lactis*	99%	MF098115.1
4. *Lactococcus lactis*	99%	MF098115.1
5. *Lactococcus lactis*	99%	MF098115.1
6. *Lactococcus lactis*	99%	MF098115.1
7. *Lactococcus lactis*	99%	MF098115.1
8. *Lactococcus raffinolactis*	99%	KC951911.1
9. *Lactococcus raffinolactis*	99%	KC951911.1
10. *Lactobacillus fermentum*	99%	MF354239.1
11. *Lactobacillus fermentum*	99%	MF354239.1
12. *Lactobacillus sp.*	99%	MH236786.1
13. *Lactobacillus sp.*	99%	MH236786.1
14. *Lactobacillus sp.*	99%	KF406344.1
15. *Lactobacillus plantarum*	99%	MG754528.1

According to the results of sequencing and phylogenetic analysis showed that the only 6 isolates of *lactobacillus* bacteria had over 99% homology with identified *lactobacillus* spp. bacteria recoded in the GenBank. In addition, the 9 isolates were identified as a *Lactococcus* spp. and had over 99% similarity with *Lactococcus* bacteria previously registered and listed in the GenBank. The Phylogenetic tree for each gene was made by Maximum Likelihood method and minimum evolution method to find the relationship of local samples with the highest query cover above 90% of samples. Results revealed the distribution of the Iraqi samples, in this figure the (S12) was extremely likely to the samples from China while both S13 and S14 show more relation to the samples from India. While, the figure (2b) reveals the distribution *lactobacillus plantarum* local strain that show more nearby with samples from China. In figure (2c) the *Lactobacillus fermentum* S10 local strain show similarity with sample from China. The S11 *Lactobacillus fermentum* local strain shares the same ancestor but it has the special branch.

The phylogenetic tree for *Lactococcus lactis* played an effective role in the distribution of the two local isolates in

the tree led to collection of the other samples with the Iraqi samples (maximum likelihood) alone in an especial group.

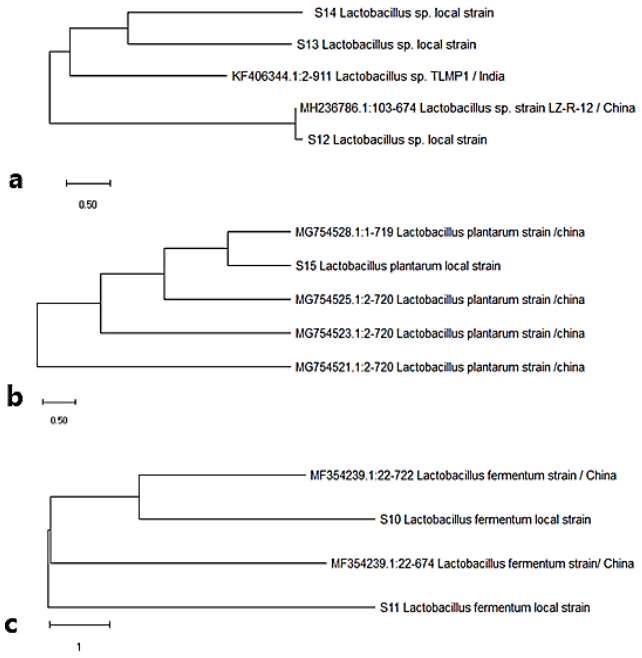

Figure 2: Phylogenetic tree analysis of a: *Lactobacillus sp*. b: *Lactobacillus plantarum*, c: *Lactobacillus fermentum* constructed based on 16S rRNA sequence analysis, showing the phylogenetic location of isolated strains from raw goat milk. The tree was constructed by the neighbor-joining statistical method, and GenBank ID of each strain used for contrast are given

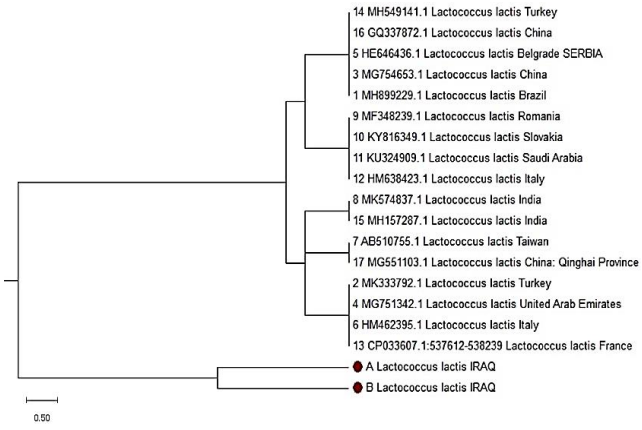

Figure 3: The Phylogenetic tree analysis of a *Lactococcus lactis* constructed based on 16S rRNA sequence analysis, showing the phylogenetic location of strains isolated from raw goat milk. The tree was constructed by the neighbor-joining statistical method, and GenBank ID of each strain used for contrast are given

While the other isolates showed the maximum similarity with strains from Turkey, China, Brazil, Saudi Arabia, India, Belgrade, Italy, Romania, and France. According to Chen *et al* (36) that the lactic acid bacteria is like any other bacteria and its capability to exchange genetic materials from the environment through the horizontal gene transfer to be more adapted and survive in the new environment. Therefore, compared with traditional identification methods 16S rRNA sequence analysis method is more accurate and more reliable, with obvious advantages in the identification of lactic acid bacteria strains at the level of species (37).

Conclusions

On the basis of the present results, it can be concluded that goat milk contains different types of bacteria, including the beneficial lactic acid bacteria which produce lactic acid and other metabolic products. The distribution of the Iraqi isolates was extremely likely to the isolates from different Asian countries.

Acknowledgements

This work was supported by the Department of Veterinary Microbiology, College of Veterinary Medicine, University of Basra, Basra, Iraq.

Conflict of Interest

The authors declare that there is no conflict of interest.

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