Isolation and Identification of Lactic Acid Bacteria from Buffalo's Raw Milk in Basrah Province by Sequencing the 16S rRNA.

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Abstract

Milk is a nutrient substance that screted from mammary glands of mammals. Lactic acid bacteria (LAB) of raw milk are natural microflora and might be starter cultures for milk fermentation. This study aimed to isolate LABs from raw buffalo's milk and identify them by 16S rRNA sequencing for possible uses in the manufacture of food product., A total of 30 buffalo's raw milk samples were collected from different areas of Basrah province (Shut-Alarab,Al-Zubair,Al-Qurna, Basrah conter and Abi-Elkhasib through the period extended from October to December 2019 to isolate the LABs using conventional bacteriology test and identify them by 16S rRNA sequencing using Polymerase Chain Reaction assay (DNA-based method). DNA extraction was carried out in duplicate for all raw milk samples. The results showed buffalo's milk raw showed positive result for bacteriological test (Gram positive-catalase negative)and for the presence of the 16S rRNA gene. There was no significant difference among ages, months, and area of the current study. Buffalo's raw milk at >4-8 year of age showed a higher ratio of LABs (71.43%) compared with that of buffalo at >1-4 year of age (47.83%). In addition, the highest ratio of LABs in buffalo's raw milk was in October (66.6%) and the lowest one was in November (36.3%). Furthermore, the highest ratio of LABs in buffalo's raw milk was in Abi- Elkhasib (100 %) and the lowest one was in AL-Zubair (20%). In conclusion Lactic acid bacteria were found in buffalo's raw milk. Biochemical and genotypic tests are effectively used to identify the LABs. All LABs isolates could be survived well in buffalo's raw milk in any age per year, months, and area of study. These findings suggest the possibility that LABs isolates from buffalo's raw milk might use to inhibit pathogenic and spoilage bacteria in the food products and might improve flavour and quality of the food products.

Key words: *Lactic acid bacteria, 16S rRNA gene, buffalo's raw milk, starter cultures and probiotics*

Introduction

Raw milk is a nutrient white fluid that comes from mammary glands of mammals. It is the main source of nutrition for infant [1]. It consists of a wide variety of bacterial species as a natural microflora of animals and human being. The majority of them belong to genera of lactic acid bacteria [2]. A lactic acid bacterium (LABs) is widely distributed in raw milk, dairy products, and decaying plant materials.

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A lactic acid bacterium is considered the most general and significant starter cultures used in fermented dairy products [3]. It has been applied in milk fermentation process worldwide [4]. Milk fermentation process has been relied on the activity of LABs which produce organic acid as the end product of carbohydrates fermentation [5]. In addition, LABs are widely used in inhibiting pathogenic and spoilage bacteria in the food products [6,7]. Furthermore, LABs in milk are used to improve of gastrointestinal disorders and prevent of certain allergies [8]. LABs used as preservation substance and improving both of flavour and quality of the food products[9]. Applications of LABs have a long history in developed countries. Significant importance of LAB in food industry and health enhancement has prompted

the developing countries to isolate and identify LABs from raw milk animals and optimize them for industrial applications. Recently, many scientists around the world (Iran, Malaysia, Egypt) have been working on LABs [3,5,6]. From this point of view, this work aimed to isolate of the LABs from raw buffalo's milk and identify them by 16S rRNA sequencing for possible uses in the manufacture of food product.

Material and Methods

Samples collection

A total of 30 raw milk samples were collected from different areas of Basrah province (Shut-Alarab,Al-Zubair,Al-Qurna, Basrah conter and Abi-Elkhasib). Before sample collection, udders were washed and dried. Then, the milk samples were collected in the sterile tubes, transferred to the laboratory in an ice box, and stored at -20°C until analysis. The samples were collected from five different regions of Basrah province through period extended from October to December 2019.

Isolation of lactic acid bacteria

For LABs isolatiion, raw milk sample (0.1 ml) was spread on the de Man, Rogosa & Sharpe agar surface (MRS agar ,Merck, Germany) and the plate was incubated at 37°C for 24 h. A loopful an overnight culture was then transfered from MRS agar, inoculated in the MRS broth (10 ml),and incubated at 37°C for 24 h. From each tube, 0.1 ml was cultured two times on the surface of the MRS agar (Merck, Germany) for further purification. The streaks plating were then incubated using anaerobic incubation jar at 37°C for 48h to provide a good environment condition for growing LABs [10]. After incubation, colonies with distinguished morphologies (white and cream colonies) was swabbed on clean slide, stained with Gram's stain, and examined under light microscope.

Biochemical test

For catalase test, a small amount of white and cream colonies was swabbed on clean slide using wooden stick and a drop of H_2O_2 was then added. Gas bubbles evolutions indicate a positive reaction [11].Colonies with distinguished morphologies (white and cream colonies) and physiologies (Gram positive-catalase negative) were collected. To identify LABs isolates, 16S rRNA gene sequences were analysed by PCR [12].

Analysis of the 16S rRNA gene sequences

DNA extraction and detection

DNA extraction was carried out in duplicate for all raw milk samples using the genomic DNA extraction kit (Qiagen- Germany). The DNA extraction kit consists of GB Buffer, W1 Buffer, Wash Buffer, and Elution Buffer. DNA extraction was performed according to the manufacturer's instruction manual in following steps: Cell harvesting, lysis, DNA binding, washing, and DNA elution. For cell harvesting: bacterial cultured (1 ml) was transferred to a microcentrifuge tube (1.5 ml), centrifuged at 8.000 rpm for 1min to discard the supernatant. Lysozyme buffer (200µl) was added to the tube to re-suspend the cell pellet at room temperature. For lysis: GB buffer (200 µl) was added to the tube in water bath at 60 °C for 10 min to lysate. For DNA binding: absolute ethanol (200 µl) was added to lysate and mixed thoroughly, transferred to the GD column in 2 ml collection tube, centrifuged at 8.000 rpm for 2min. For wash step: W1buffer (400 µl) was added to the GD column in 2 ml collection tube and centrifuged at 8.000 rpm for 30 sec to dry the column matrix. Then, wash buffer (600 µl) was added to the GD column in 2 ml collection tube and centrifuged at 8.000rpm for 30 sec to dry the column matrix. For DNA elution: The dried GD column was transferred to microcentrifuge tube (1.5 ml). Pre-heat elution buffer (100 µl) was then added to the center of the column matrix, stand for at least 3 min to ensure the elution buffer absorbed by the matrix, and centrifuged at 8.000 rpm for 30 sec to elute the purified DNA.

PCR primers and amplification

The primers described in previous study (F:5'-GCGGCGTGCCTAATACATGC -3'; R:5'-ATCTACGCATTTCACCGCTAC -3') were used to amplify a 16S rRNA gene sequences (700bp)(12,13)

Polymerase Chain Reaction assay for gene amplification

DNA from the samples was amplified in a total reaction volume (25 µL) containing genomic DNA (1µg), primers (1µM), Mgcl2 (2mM), dNTP (0.2mM), PCR buffer (2.5µL of 10X) and the enzyme Taq DNA polymerase (1unit). The Polymerase Chain Reaction (PCR) assay was done as follows: Initial denaturation step (95°C for 5 min) followed by 30 cycles of: Denaturation step (95◦C for 1 min), annealing step (42°C for 1 min),

extension step (72°C for 1 min), and a final elongation (72◦C for 10 min) using a thermocycler(Techne-UK) (12,13).

PCR products detection The amplified PCR product was detected on agarose gels (1.5%) prepared with agarose in Tris-borate-EDTA buffer (1x), stained with a fluorescent stain, and images by gel-documentation systems. The size of the band was selected by comparison with a standard (100 bp) DNA ladder [14].

Statistical analysis The Pearson's chi-square test was done by using statistical program, SPSS [15]

Results and discussion In the current study, the LABs was isolated from buffalo's raw milk, identified through conventional bacteriology and genotypic tests, and investigated their distribution rates based on age per year, months, and area of study.

Biochemical test

In the present study, the isolation results of LABs from buffalo's raw milk using MRS medium revealed that 16 out of 30 (53.3%) were positive for Gram stain, negative for catalase test, and live under anaerobic condition. This finding is in agreement with previous study in which that conventional phenotypic method (biochemical reactions-based method) using MRS medium is suitable to identify the LABs in the breast milk and both of cow and goat raw milk providing a reliable identification of the isolates [13] .

Polymerase Chain Reaction (PCR) assay

In the current study, 16 out of 30 (53.3%) buffalo's raw milk showed positive result for the 16S rRNA gene. The amplification length of the 16S rRNA gene was 700bp (Figure 1). Gene sequences (16S rRNA) for the identification of buffalo's raw milk was effectively done by using PCR. This finding is in agreement with previous study, in which that sequencing of the V1 region (700 bp and 90 bp) for the gene sequences (16S rRNA) are sufficient to identify the LABs in the breast milk and both of cow and goat raw milk providing a reliable identification of the isolates [16]. It has been found that molecular method (DNA–based method) is possibly accurate for identification of bacteria [17].

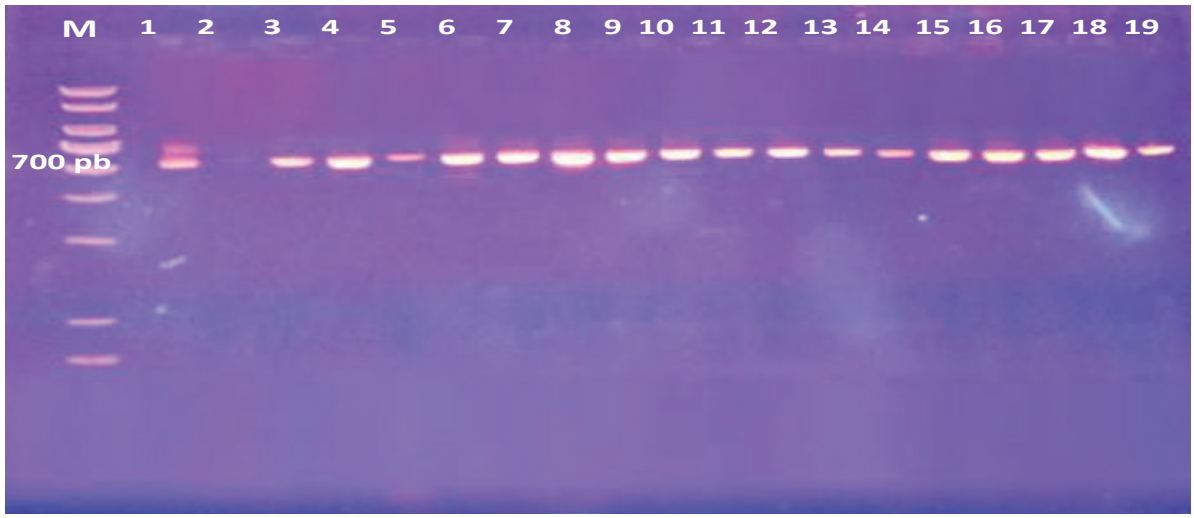

Figure 1: The amplification of the 16S rRNA gene (700bp) by using PCR in agarose gel electrophoresis of DNA. M: ladder (100 bp); lane (1-19) raw milk samples.

Distribution of LABs in buffalo's raw milk based on age per year

The difference among ages showed that there was no significant difference (P>0.05) of LABs in buffalo's raw milk. Raw milk of buffalo at age >4-8 year showed a higher ratio of LABs (71.43%) compared with that of buffalo at age >1-4 year (47.83%)(Table1). This finding is in agreement with previous study, in which ages did not effect on the LABs in goat's raw milk (13).However, this finding disagreement with previous study, in which ages effect on the LABs in cow's raw milk. It has been found that the number of parturition significantly effect on the LABs distribution in cow's raw milk [13]

Table 1: Distribution of LABs in buffalo's raw milk based on age per year

Age /year	Examined N.	Positive N.	percentage
>1-4	23	11	47.83%
>4-8	7	5	71.43%
Total	30	16	53.33%
Chi-Square(df=1)=1.20; P value =0.27			

Distribution of LABs in buffalo's raw milk based on months study

The difference among months showed that there was no significant difference (P>0.05) of LABs in buffalo's raw milk. Raw milk of buffalo revealed the highest ratio of LABs was in October (66.6%) and the lowest one was in November (36.3%) (Table 2). This finding disagreement with previous study, in which there is a seasonal variation during the long study period (January till December, 2014) in the microbial composition and quantity of cow's raw milk compared with the short present study period (October till December)[18].

Table 2: Distribution of LABs in buffalo's raw milk based on months study

Months	Raw milk		
	Examined N.	Positive N.	Percentage
October	9	6	66.6%
November	11	4	36.3%
December	10	6	60%
Total	30	16	53.3%
Chi-Square(df=2)= 2.09; P value =0.35			

Distribution of LABs in buffalo's raw milk based on region of study

The difference among regions, in which buffalo's raw milk was whereabouts, revealed that there was no significant difference (P>0.05) of LABs in buffalo's milk. Raw milk of buffalo revealed the highest ratio of LABs was in Abi- Elkhasib (100 %) and the lowest one was in AL-Zubair (20%) (Table 3). This finding is in agreement with previous study, in which the region studies did not effect on the LABs in raw milk [13].

Table 3: Distribution of LABs in buffalo's raw milk based on regions of study

Region	Raw milk		
	Examined N.	Positive N.	Percentage
Shut-Alarab	4	1	25%
AL-Zubair	5	1	20%
AL-Qurna	12	7	58.3%
Basrah center	6	4	66.6%
Abi- Elkhasib	3	3	100%
Total	30	16	53.3%
Chi-Square(df=4)=6.69; P value = 0.15			

Conclusion

A lactic acid bacterium was found in the buffalo's raw milk. Biochemical and genotypic tests are effectively applied to identify the LABs. All LABs isolates could survive well in buffalo's raw milk based on any age per year, months, and area of study. These findings suggest the possibility that LABs isolates from buffalo's raw milk might use as probiotics (live bacteria that are halpful for human beings) and improve flavour and quality of the food products and might use as preserve agents

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