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Isolation and Identification of Lactobacillus Using Biochemical and Molecular Methods

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Abstract: The present study includes isolation of fifteen isolates of lactic acid bacteria (LAB) from animals milk (cows, sheeps and human's milk). These isolates were identified using morphological and biochemical tests, the results revealed that all the isolates belong to the *Lactobacillus* genus. In addition, the genetic variations were analyzed among these bacterial isolates by polymerase chain reaction technique random ampilification of polymorphic DNA (RAPD PCR) and the results, showed that diversity among these isolates exist at high level which may be related to the source of these bacteria.

Keywords: lactic acid, Bacteria, RAPD-PCR, *Lactobacillus*.

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Introduction:

Lactic acid bacteria (LAB) could be isolated from dairy products, fermented foods, plants, soil, water, silages, and waste products and also from the intestinal tract of animals and humans. Lactic acid bacteria are probiotic microorganisms extensively studied for their commercial potential, food preservation and health benefits. They are industrially important used worldwide in the dairy industry for manufacturing fermented milk products and cheese. Industrial importance of LAB is based on their ability to ferment sugars readily into different metabolites and provide an effective method for preserving fermented food products (1).

Moreover, although many members of LAB are perfectly safe and used for generating in food.

These bacteria is a group of gram positive rods and cocci and non-spore forming, occurring naturally in a variety of niches (2,3).

This group of bacteria are widely recognized genera which include: *Carnobacterium, Entercoccu, Lactobacillus, Lactococcus, Leuconostoc, Oenocococcus, Pediococcus, Streptococcus, Tlerenococcus, Vaocococus, Weissella*(4).

Identification of LAB based on carbohydrate fermentations patterns is unreliable and not accurate enough to distinguish closely related strains due to their similar nutrition. The traditional methods for identifying bacteria rely on morphological, physiological and biochemical criteria which are generally laborious and time consuming (5).

Nowadays, the main focus for the identification has moved from phenotypic to genotypic methods as their yield more sensitive and accurate results of these is the polymerase chain reaction based on the amplification of random fragments of DNA (RAPD has

been used to determine the diversity of LAB food (6,7).

In the present study, the classical and RAPD-PCR method were applied to taxonomic identification and evaluation of genetic diversity within LAB isolated from different source of animals milk in Mosul city (Iraq).

Materials and Methods:

Isolation of LAB:

A total of 25 unpasteurized milk samples were collected from cows and sheep cattle breeding field of agricultural college Mosul university, as well as from human breast newborn under aseptic conditions in sterile screw cap tubes. These samples were brought directly in a sterile bag to the laboratory of molecular genetics research's for living organisms in biology department education collage Mosul university. Milk samples were inoculated in a diluted nutrient broth medium and then incubated at 3°C for 20 minutes before plating for increasing the number of bacteria.

Diluted samples were plated on to selective medium Man Rogosa Sharpe (MRS) for LAB isolation and incubated at 37 °C for 48-72h. After incubation, isolated colonies were taken randomly to be purified. The obtained purified colonies were examined for LAB by microscopic examination using Gram stain (8).

Biochemical identification:

All isolates were identified according to their morphological, cultural, physiological and biochemical characteristics (9,10). Production of catalase, carbohydrate fermentation, growth at different temperature ,methyl red, indole production in peptone water medium, urease activity, gelatin liquefaction were used. As described by Berge's Manual of Systematic Bacteriology.

Molecular analysis:

Isolation of genomic DNA:

Genomic DNA from all the isolates were extracted as described by Wilson(11).

RAPD-PCR:

The RAPD -PCR reactions were done for all LAB isolates using 7 primers (Bioneer) whose numbers and sequence are shown in (Table 1).

Table (1): Numbers and sequences of the RAPD primers used.

No.	Primer	Sequences 5' → 3'
1	OPW-11	CTGATGCGTC
2	OPA-1	CAGGCCCTTC
3	OPA-2	TGCCGAGCTG
4	OPA-13	CAGCACCCAC
5	OPA-4	AATCGGGCTG
6	OPW-13	CAGCACCCAC
7	OPH-18	GAATCGGCCA

Amplification reaction solution were performed in an eppendrof PreMix (Bioneer) following the method described by Sajjad *et al*. (12).

Table (2): Composition of PCR PreMix (bioneer) reaction.

Composition	50µl of reaction
Taq DNA Polymerase	2.5U
Primer	10 pmoles
DNA sample	50ng

The conditions of the thermal cycle were carried out according to following program (12):

1- Initial denaturation at 94°C for 5 min.
2- 35 cycles which include: denaturation at 94°C for 1min, annealing at 35°C for 1min, and extension at 72°C for 2min.
3- Final extension at 72C for 5min After the program ended, amplification products were loaded in 1.2% agarose, and the gel was running in TBE (1X) buffer for 15 min at 45V and3h at 70V, then illuminated by UV-trans illuminator at 320 nm and photographed by digital camera.

Analysis of the amplified DNA polymorphisms:

The banding patterns on the gels were transformed into tables of binary characters where appearance of a band was given the numbers one (1) while the absence of the band was denoted by zero (0). The tables used to determine the total number of bands together with their sizes that produced by a primer across all were isolates.

To determine % efficiency of primer, this was estimated as percentage of the total number of bands amplified by the primer out of the total number of bands amplified by all primers across all species(13). But, the discriminatory power of the primer, represented by the percentage of the polymorphic bands amplified by a primer out of the total number of polymorphic bands given by all primers in all isolates. The tables of binary characters can be used to determine the genetic distances between the various species which were fed into the computer program Similarity for Qualitative Data (SIMQUAL). The resulting matrix of the genetic distances is used to draw the clustering of the *Lactobacillus* isolated from animals milk within a dendrogram depending on the genetic closeness of members. This was achieved by applying the data on the Weighted Pair –Group Method with arithmetic (UPGMA). All these were with the statistical package: MVSP program version 3.22.

Results:

All the 15 isolates understudy were found to be gram positive, and rod shape arranged in pairs or chains. Their colonies on MRS medium were found to be circular, rough surface, low convex and white colored, these isolates were determined as representative of the genus *Lactobacillus* (14).

Also the isolates were tested for fermentation of glucose, fructose, lactose, manitol, sucrose and maltose. It is clear from the table that there is diversity revealed by the isolates understudy in fermentation of sugars used (Table 3), the isolates LAB1, 2, 3, 7, 11, 12, 14,15(8 isolates) give positive results for glucose, fructose, lactose. While LAB5, 8, 9, 10 (4 isolates) gave

positive test results with glucose, fructose, lactose, sucrose, maltose. In addition LAB4(1 isolates) give positive result for glucose, fructose, lactose, mannitol, while LAB6 give positive result for glucose, fructose, lactose, mannitol, and sucrose. LAB13 give positive results for glucose, fructose, lactose, maltose. All isolates grew at 10, 15, 37, 54°C. Moreover, all isolates were catalase negative test ability to produce indole, they were observed unable to produce indole and all isolates give positive result for methyl red except one and finally all of them could not liquefy gelatin.

According to the results obtained from biochemical and physiological tests, these isolates can be classfied to many species. It seems that LAB1, 2, 3, 7, 11, 12, 14, 15(53.3%) from cows and sheeps may be like to *Lactobacillus bulgaricus* which isolated and LAB5, 8, 9, 10 (26.6 %) may be belonged to *Lactobacillus lactis* isolated from human breast milk.While LAB4 (6.6%) seem to be identified to *Lactobacillus acidophilus*, LAB6 (6.6%) belong to species *Lactobacillus plantarum*, LAB13(6.6%) may be to species *Lactobacillus casei* (15,16).

Table (3): Morphological, biochemical and physiological characteristic.

Morphological biochemical	COWS MILK								Human milk		Sheep milk				
	LAB 1	LAB 2	LAB 3	LAB 4	LAB 5	LAB 6	LAB 7	LAB 8	LAB 9	LAB 10	LAB 11	LAB 12	LAB 13	LAB 14	LAB 15
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Colony surface	Rough cottony	Rough Cottony	Rough Cottony	Rough	Rough	Rough	Rough Cottony	Rough	Rough	Rough	Rough Cottony	Rough	Rough Cottony	Rough Cottony	Rough Cottony
Colony color	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White
Catalase test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentatin															
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Mannitol	-	-	-	+	-	+	-	-	+	-	-	+	-	-	-
Sucrose	-	-	-	-	+	+	+	+	+	+	-	+	-	-	-
Maltose	-	-	-	-	+	-	+	+		+	-	-	+	-	-
Growth at 10,15,37,45°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Urease test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin liquefactin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Molecular analysis:

The method of Wilson (11) for extracting genomic DNA from LAB bacteria gave a good yield of genomic DNA at a concentration of 27-47 µg/ml for each 1.5 ml of bacterial sample. The purity of the DNA preparation ranged between (1.6-1.8). The concentration of genomic DNA was diluted to 50ng for PCR reaction set by Sajjad *et al*. (12). which appear to be shown on (Figure 1).

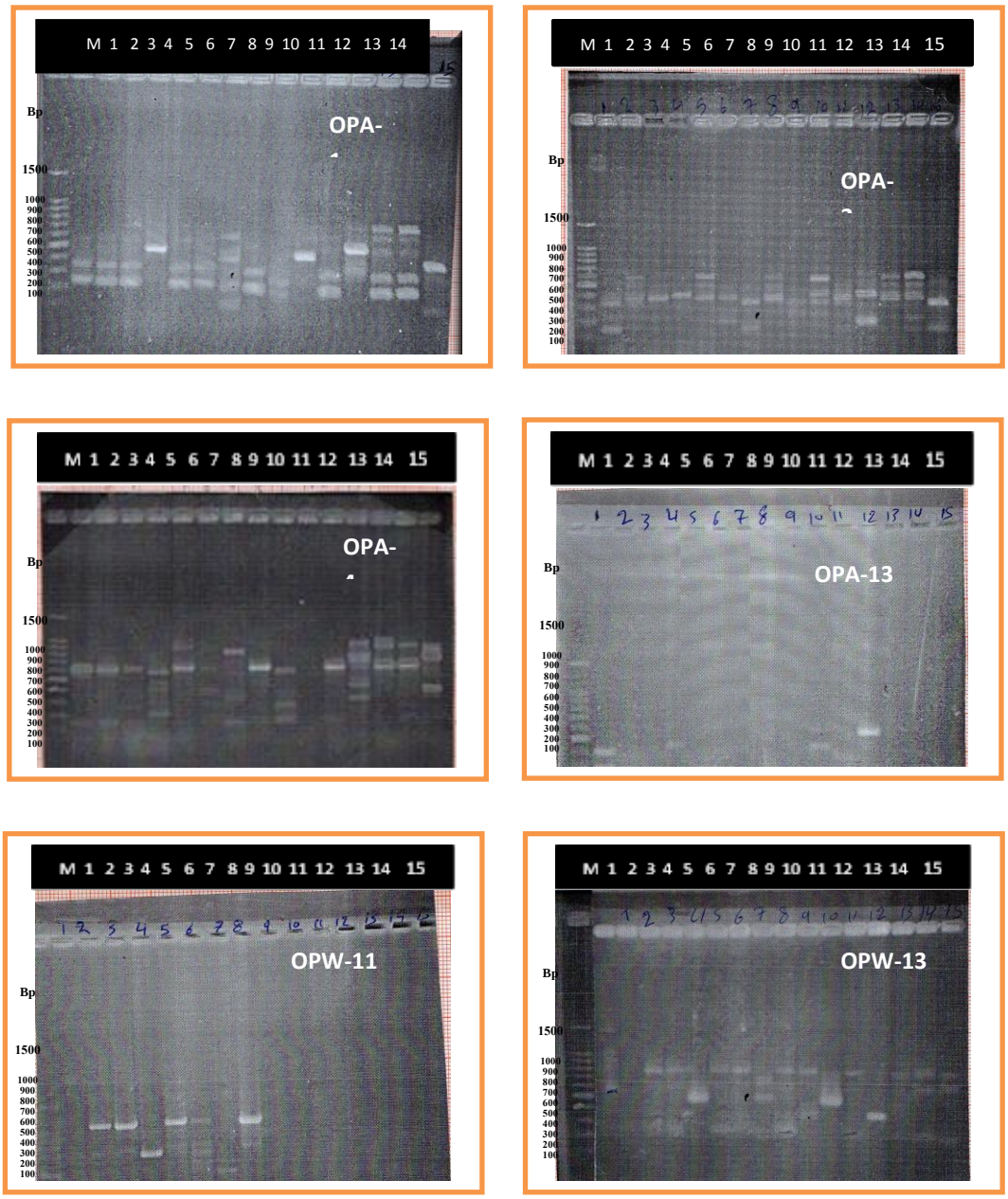

Figure (1): Products of RAPD PCR reactions in agarose gel electrophoresis (1.2) using random primers.

All the used primers used gave positive reactions except one (OPH-18 that no gave amplified fragments generated was this might be attributed to lack of complementary sequence to this primer among the genomic DNA(17).

The banding patterns on the gels were transformed in to table of binary characters. The obtained results of RAPD- PCR using 6 primers were summarized in (Table 4).

Table (4): DNA amplified fragments by the 6 primers in the 15 bacterial species and the % efficiency of amplification and discriminatory power of each primer.

Primer	Sequence 5→3	Number of bands amplified in all 15 spices		Primer efficiency (%)	Primer discriminatory power %
		Total	polymorphic		
OPW-11	CTGATGCGTC	26	9	18.8	19.14
OPA-1	CAGGCCCTTC	31	8	22.4	47.57
OPA-2	TGCCGAGCTG	27	13	19.5	41.48
OPA-13	CAGCACCCAC	12	4	8.69	8.51
OPA-4	AATCGGGCTG	16	7	11.59	24.56
OPW-13	CACAGCGACA	26	6	18.8	48.51
Total number		138	46		

From the above table, it is clear that 6 (85.7%) primers gave amplified bands totaling at 138 across the 15 bacterial species, while the polymorphic bands are 46 bands. Also, we noticed that OPA-1 was a high efficient primer (22.4%), while the primer discriminatory of the same primer was 47.57 % , the lowest efficiency primer OPA-13 (8.69%) and its discriminatory value was (8.51%).

High efficiency of a primer is indicative of a large area of the genome that complement and allows base pairing between the primer and the genomic DNA (18).

Polymorphic bands of fragments were also useful in the genetic similarity between species and estimating the genetic distance between each pair of species (19).

Statistical analysis:

The resulting of RAPD –PCR product for LAB isolates were transferred into tables of binary characters of genomic DNA bands and the average of genetic distance was determined by the distance of matrix using percent similarity , the results was showed in (Table 5).

Table (5): Matrix of genetic distance between the 15 *Lactobacillus* species as deduced from their RAPD markers.

UPGMP
Jaccard's coefficient
Similarity matrix

	LAB1	LAB2	LAB3	LAB4	LAB5	LAB6	LAB7	LAB8	LAB9	LAB10	LAB11	LAB12	LAB13	LAB14	Lab15
LAB1	1.000														
LAB2	0.400	1.000													
LAB3	0.300	0.225	1.000												
LAB4	0.625	0.450	0.573	1.000											
LAB5	0.608	0.545	0.600	0.286	1.000										
LAB6	0.400	0.400	0.625	0.654	0.700	1.000									
LAB7	0.273	0.429	0.357	0.513	0.438	0.633	1.000								
LAB8	0.499	0.700	0.444	0.250	0.100	0.750	0.733	1.000							
LAB9	0.444	0.500	0.511	0.300	0.167	0.422	0.633	0.222	1.000						
LAB10	0.467	0.567	0.583	0.354	0.133	0.477	0.676	0.167	0.000	1.000					
LAB11	0.250	0.364	0.200	0.431	0.500	0.500	0.600	0.667	0.500	0.450	1.000				
LAB12	0.000	0.067	0.000	0.463	0.588	0.567	0.048	0.643	0.683	0.443	0.063	1.000			
LAB13	0.454	0.250	0.573	0.331	0.500	0.600	0.613	0.667	0.400	0.550	0.600	0.214	1.000		
LAB14	0.250	0.264	0.400	0.331	0.636	0.667	0.300	0.875	0.500	0.550	0.278	0.133	0.778	1.000	
LAB15	0.231	0.143	0.154	0.433	0.688	0.531	0.375	0.731	0.600	0.531	0.214	0.125	0.308	0.308	1.000

It is obvious from the table that the longest distance was 0.875 separating between the two isolates Lab8 (human milk) and LAB14 (sheep milk) while smallest distance is between LAB7 (Cow's milk) and LAB12 (sheep milk) with a genetic distance of only 0.048 (Table 5). It was showed that the low value of genetic distance between species indicates that genetic diversity among these species was low, while a high value of genetics distance between isolates indicates a high genetic diversity among isolates. Also it is clear from the table, there was a genetic distance 0.00 between LAB1 (cow milk) and LAB12 (sheep milk), LAB3 (cows milk) and LAB12 (sheep milk), which indicates that these are closely related to each other. The remaining genetic distance were between these two limits was shown in (Table 5). So, clear genetic variation appear to be present among the isolates understudy and as expected to be because they are isolated from different sources and specific PCR may give more information of analyzed genetic variations. These distances are used to produce the clustering dendrogram of 15 isolates. A conclusion could be deduced from the above results that the diversity among bacterial isolates understudy could be related to genetic mutation in the DNA sequence of the genomic bacteria. Genetic distance was determined between 15 lactic acid bacteria using MVSP-version 3.22.

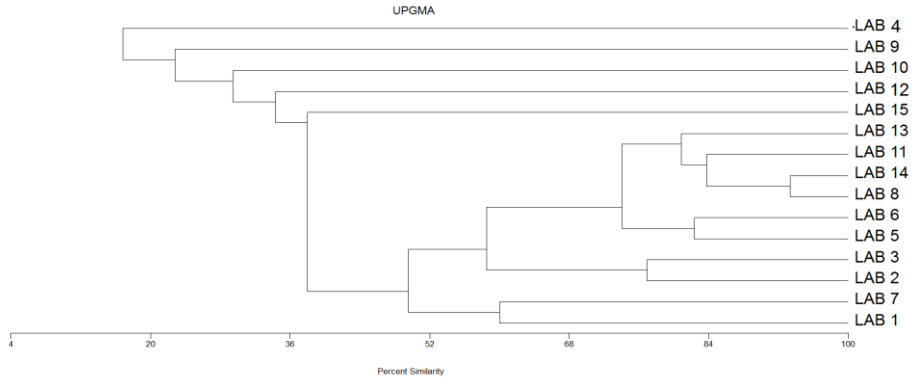

Figure (2): Dendrogram of 15 isolates belong to *Lactobacillus species* amplified by 6 RAPD primers.

It is obvious from (Figure 2), that there are 14 nods which included 15 LAB isolates belong to *Lactobacillus* genus and has different value of genetic distance, so these isolates included two groups.

Although the low number of primers used in this study but these LAB isolates classified within groups and these groups have different values of genetic distances, and most of these genetic distance between LAB isolates was more than 0.50 and the table 4 shows the results.

Discussion:

From the results obtained that our LAB isolates are more available in cows milk followed by sheep milk and human milk which agree with Aziz *et al*., (20) isolated LAB from cows and sheeps milk, reveald that the percent incidence of LAB was highest in cows milk followed by sheeps milk, four species identified in cows milk sample these are *L. lactis , l. bulgaricus , L. cremoris* and *Streptococcus thermophiles*.

Also the results are in concordance with Bassyouni *et al*., (21) who isolated lactic acid bacteria from dairy products in Egypt using morphological and biochemical analysis. They indicated that the isolated bacteria related to the genus *Lactobacillus* and suggested that they were belonged different species of these *L.casei*(4 isolates), *L.acidophillus* (3 isolates) nd *L.lactis* (1 isolates).

Mithum *et al*. (22) isolated 163 colonies from raw milk samples in Mumbia city, they identified these colonies using physiological cultural and sugar fermentation patterns test, all of the 163 isolates were confirmed to belong to the genus *Lactobcillus*: *Lb.fermentum* (48%), *Lb.acidophilus* (34%), *Lb. viridescens* (8%), *Lb.brevis* (5%) and *Lb. grasseri*.

Thirty-eight colonies of LAB from a total of 40 samples of milk were collected by Bhardwaj *et al.*, (23). These colonies were subjected to cell morphology, physiology and array of biochemical characterization, they found that all isolates were confirmed to different spices of *Lactobacillus*: *Lb. casei* (24.35%) , *Lb.brevis* (3.84%) , *Lb. fermentum* 6.41%, *Lb plantarum* 7.69%, *Lb. lactis* 3.84 %, *Lb. acidophilus* 37.17%.

In Rawalpindi region Toqeer *et al*. (24) isolated *Lb. lactis and Lb. acidophilus* from camel milk and reported that *Lb.acidophilus* grew relatively milk. more rapidly in camel milk. Fifty sample of indigenous dahi were collected randomly from local market of Islamabad by Masud *et al*. (1991)(25) to determination the incidence of LAB, the bacterial isolates were *Lb*. bulgaricus (86%), *streptococcus thermphilus* (80%), *Streptoco ccus lactis* (74%), *Lb. helveticus* (34%), *Lb.caseiv* (20%) and *Lb.acidophilus* (14%). Our results agree with the above work concerning the availability of the obtained isolated species in milk sample in the above work and disagree with their percentage of their isolation.

Pulido *et al*. (26) isolated 132 LAB at different times of fermentation of capers fruits, the collection was reduced to 75 after using RAPD-PCR analysis, the isolates were identified to 37 isolates as Lb. *plantarum*, 1 isolates (*Lb. paraplantrum*), 5 isolates (*Lb. pentosus*), 9 isolates (*Lb. brevis*), 6 isolates (*Lb. fermentum*), 14 isolates *Pediococcus pentosaceus*. The cluster analysis of RAPD–PCR patterns reveled a high degree of diversity among *Lactobacillus* with four major groups and subgroups while *pediococcus* clustered in two closely related groups.

RAPD-PCR assay combined with 16s rDNA sequencing analysis were used to describe the microbial LAB diversity of several donkey farms milk in the north west part of Italy by Soto *et al*. (27). They found that more they found that more frequently detected species were: *Lb. parcasei* and *Lb. lactis* and less abundant genera were *Leuconostoc*, *Enterococcus* and *Streptococcus*. Valcheva *et al*. (28) isolated 20 isolates from fresh wheat sourdough, these isolates were identified using biochemical and molecular tests using RAPD-PCR and 16S rRNA , all isolates members were identified to the *Lactobacillus* genus, they identified as representative to *L. plantarum, L. paralimentarius, L. sanfranscensis, L. spicheri* and *L. casei*.

RAPD –PCR was used to identified *Lactobacilii* by Andrighetto *et al*. (2001)(29), they used two primers M13 and D8635 for differentiation of 53 isolated from traditional fermented

sausages and artisanal meat plants in region (Italy), most of the isolates assigned to the species *L. sakei* and *L. currvatus* and *L. lactis*, Schillinger *et al*. (30) used specific PCR and RAPD-PCR analysis to identified species of *Lactoibacillus* genus, they found that most isolates belong to *L.casei* and *L. acidophilus* and *L. bulgaricus*.

Conclusion:

The conclusion could be made that *Lactobacillus* are present in diary product such as milk. Conventional methods of detecting and determining the species of lactobacilli from milk is time –consuming and often ambiguous. Recently, research has focused on the application of molecular methods such as PCR for rapid detection and differentiation of LAB. RAPD–PCR technique used in this study is a highly efficient method for evaluating the diversity. Although the low numbers of primers used in this study, but there are a diversity in our data of LAB isolates understudy which isolated from different sources of animals milk.

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