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Original Article

Identification of *Lactobacillus* Species Isolated From Traditional Dairy Products Using RAPD-PCR

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Abstract

Background: Probiotics are non-pathogenic useful microorganisms having positive effects on the host health. The aim of the present study was to discriminate *Lactobacillus* species extracted from traditional dairy products.

Methods: This study was conducted on 26 specimens collected from traditional dairy products in Bukan. *Lactobacillus* species were separated and purified employing biochemical tests. Then, the intra/inter-species diversity was investigated using RAPD-PCR (random amplified polymorphic DNA-polymerase chain reaction) technique.

Results: Polymorphism information content (PIC) value varied between 15.9% and 34.4% with a maximum value of 34.4% associated with primer 1254. The mean of and Marker index (MI) for the 6 primers was 4.52, in which the maximum and minimum values belonged to the primers 1254 and OPA-02, respectively. The isolates were categorized into 4 main clusters according to Jaccard similarity coefficient using UPGMA (unweighted pair group method with arithmetic means) clustering method. Principle coordinates analysis (PCoA) demonstrated that the first and second components explained 30.59% and 22.48% of variances, i.e. 53.07% of variances in total. The results of RAPD marker indicated that the intra-species diversity was greater than inter-species diversity. The intra-group variance explained 94% of the all variance, while inter-group variance explained only 6% of the all variance. Moreover, the results of analysis of molecular variance (AMOVA) indicated that the highest level of discrimination occurred at the 16 groups cut-off point with a similar coefficient of 0.56.

Conclusions: From the results of the present study, it can be concluded that traditional dairy products are enriched sources of probiotic bacteria which can ensure the health of general population and enhance their immunoe systems. Moreover, RAPD-PCR is an appropriate method for detection and calssification of lactobassiluses.

Keywords: Probiotic, *Lactobacillus*, RAPD-PCR

Background

According to the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), probiotics are viable microorganisms providing the human body with many benefits if consumed sufficiently (1). Probiotics are generally of human resources and known as non-pathogenic bacteria (2,3). The most common bacteria used as probiotics are driven from lactic acid bacteria family and can normally be found in dairy products such as yoghurt, cheese, etc. Traditional dairies are full of such organisms. Naturally, the lactic acid bacteria are residents of the human gut and have a long history in fermentation products (4). The genus *Lactobacillus* bacterium, as a lactic acid bacterium, has attracted more attention than do other species of the family (5).

According to the definitions of International Dairy Federation (IDF) and the International Organization for Standardization (ISO) in 2008, the lactic acid bacteria are gram-positive, immobile, non-spore forming, catalase-negative, negative nitrate reduction, and cytochrome oxidase negative. All of them have fermentative metabolism and are strongly saccharolytic. The most important lactic acid bacteria in the dairy industry belong to the *Pediococcus*, *Lactobacillus*, *Leuconostoc*, *Lactococcus,* and *Enterococcus* species (6).

Although such bacteria have many advantages for human body, they may be missing in industrially-produced dairy products, so it seems to be necessary to detect and extract such bacteria from traditional products and to utilize them in products manufactured by large industrial plants. The results of the studies conducted in this area have demonstrated that traditional dairy products contain more probiotics than do their industrially-made counterparts. As a result, it would be beneficial to add probiotics to the industrially-made cheese and yoghurt in order to produce high quality dairies with characteristics similar to traditional ones. From the marketing point of view, moreover, probiotic enriched dairies and foods are more popular (7,8).

For decades, the detection of lactic acid bacteria had been performed using phenotypic methods such as cellular morphology, differences in carbohydrate substrates, and growth rates in various temperatures. The lack of reproducibility of results is the main problem associated with phenotypic methods, because one result obtained by a laboratory could not be reproduced by another. Moreover, biochemical methods are not able to discriminate lactobacilli with similar morphologies (9). Accordingly, it is of pivotal importance to detect bacteria species with molecular-based techniques, because such techniques are based on the analyses of nucleotide acids which are reproducible. Furthermore, the field has been significantly progressed during recent years, making it possible to categorize bacteria using new criteria which are based on a specific sequence of genomes as a DNA barcoding. Most molecular studies conducted on prokaryotes have utilized the 16S ribosomal RNA (rRNA) gene sequence. Molecular methods utilized so far to categorize and analyze lactic acid bacteria are: DNA–DNA hybridization, DNA sequencing, Polymerase chain reaction (PCR), and PCR-RFLP (PCR- restriction fragment length polymorphism) which is also used for determination of genus and species of the bacteria. In addition to being able to determine the genus, random amplified polymorphic DNA (RAPD), REP-PCR, AFLP, and PFGE have also the ability to specify the species (10). These methods benefit from various advantages, including reproducibility, diversity, accuracy, and being less time-consuming (11).

Objectives

According to the above-mentioned issues, the present study was set to isolate the *Lactobacillus* species from traditional dairies, produced in Bukan, using RAPD-PCR molecular marker.

Methods

Specimen Collection and Bacteria Isolation

Generally, 26 specimens were collected from traditional dairy products manufactured in Bukan (during September 2014 to June 2015). In the present study, a procedure proposed by Calicchia et al (12) was used for preparing and making the primary suspension. Because of the insolubility of the products in the peptone based solution, 2% trisodium citrate solution was employed to make the bacterial suspensions from cheese, Lurk, and Siraj samples. Petri dishes were incubated in the anaerobic condition at 37C for 24 hours. After this period of incubation, bacteria were transferred from De Man, Rogosa, and Sharpe (MRS) medium to the new solid medium. Colonies with different morphologies (with respect to their shape, color, and size) were transferred to the new medium. Afterwards, sub-cultures were provided from the suspicious colonies. Then, the refined colonies were transferred to the 10 mL MRS liquid medium. Finally, the samples were incubated for 24 hours at 37ºC and stokes were provided.

First, the specimens were cultured on the MRS medium for 24 hours, then their DNA were extracted using the method applied by Renouf et al (13). The RAPD-PCR reaction for investigating the inter-species diversity was carried out on 26 *Lactobacillus* specimens extracted from traditional dairies using 6 different primers (Table 1) with the final volume of 25 µL containing 13.5 µL PCR master mix, 1 µL DNA template, 1 µL primer, and 9.5 µL dH2O. The amplified products were electrophoresis on 1.5% agarose gel (14).

Investigating the Inter-species Diversity by RAPD-PCR Technique

The RAPD-PCR reaction for investigating the inter-species diversity was carried out on 26 *Lactobacillus* specimens extracted from traditional dairies using 6 different primers (Table 1) with the final volume of 25 µL containing 13.5 µL PCR master mix, 1 µL DNA template, 1 µL primer, and 9.5 µL dH2O.

Data Analysis

The RAPD bands were scored based on their presence (1) or absence (0) and each bond was regarded as a locus. The polymorphism information content (PIC) and Marker index (MI) were calculated using equations 1 and 2, respectively (15,16).

$PICi = 2.fi. (1-fi)$ Eq. 1; and $MI = PIC.N.\beta$ Eq. 2

Where, N denotes the total number of bonds for each primer and β represents the proportion of polymorphic markers, calculated by determining the polymorphic loci (np) and non-polymorphic loci (nnp) as $\beta = np/ (np+nnp)$. Furthermore, the genetic parameters of population

Table 1. The Primers Used for Assessing the Gene Diversity of *Lactobacillus* Species

Primer	Sequence 5' → 3	No. of Nucleotides	Ref.
OPA-02	TGCCGAGCTG	10	(36)
CRA 23	GCGATCCCCA	10	(36)
OPL-02	TGGGCGTCAA	10	(36)
P_8	CGTACAGGCT	10	(36)
$M_{13}V$	GTTTTCCCAGTCACGAC	17	(36)
1254	CCGCAGCCAA	10	(36)

such as the number of observed alleles (Na), the number of effective alleles (Ne) (17), the gene diversity index (18), and Shanon's information index (I) (19) were computed using GENALEX 6.2 software; moreover, genetic similarity and distance were calculated by the Nei method (20) and using POPGEN 1.32 software (21). Finally, dendrogram was depicted based on the UPGMA method.

To investigate the intra and inter group gene diversity AMOVA (the Analysis of Molecular Variance) method (22) was employed using GenAlEx software, version 6.4 (23). The two-dimensional graph of the individual distribution (PCA: principle component analysis) (24) was also depicted by the same software. The cophenetic correlation coefficient and Mantel test (25) were employed to investigate the correlation between the similarity matrices and final dendrograms based on Jaccard (26) and Dice, and simple matching coefficients were calculated using NTSYSpc software, version 2.02 (27).

Results

Result of Collecting Specimens and Extracting Bacteria

A total number of 26 bacteria were isolated. The location where these isolates were taken from and their sources are presented in Table 2. Besides, an identifier was assigned to each isolate (the leftmost column in Table 2).

Table 2. The List of Isolate, Their Location and Source Names

Strain	Location and Source of Isolation
G.L2	Bukan - Qaletapeh (Cheese)
G.L15	Bukan - Qaletapeh (Lurk)
G.L19	Bukan - Qaletapeh (Lurk)
G.L20	Bukan - Qaletapeh (Siraj)
G.L22	Bukan - Qaletapeh (Siraj)
G.L24	Bukan - Qaletapeh (Siraj)
G.L25	Bukan - Qaletapeh (Curd)
G.L26	Bukan - Qaletapeh (Curd)
B.L3	Bukan - Bathche (Cheese)
B.L7	Bukan - Bathche (Yoghurt)
B.L8	Bukan - Bathche (Yoghurt)
B.L13	Bukan - Bathche (Lurk)
B.L18	Bukan - Bathche (Lurk)
P.L1	Bukan- Pashblagh (Cheese)
P.L6	Bukan- Pashblagh (Cheese)
P.L21	Bukan- Pashblagh (Siraj)
P.L23	Bukan- Pashblagh (Siraj)
GA.L4	Bukan - Qaderabad (Cheese)
GA.L5	Bukan - Qaderabad (Cheese)
GA.L11	Bukan - Qaderabad (Yoghurt)
GA.L12	Bukan - Qaderabad (Yoghurt)
GA.L14	Bukan - Qaderabad (Lurk)
GA.L16	Bukan - Qaderabad (Lurk)
GA.L17	Bukan - Qaderabad (Lurk)
B.L9	Bukan- Bathche (Yoghurt)
B.L10	Bukan- Bathche (Yoghurt)

The Comparison of DNA Segments Produced by RAPD Reagent

The 6 primers used in this study produced appropriate multi-shape bands encoded within a range from 200 bp to 3000 bp. A total number of 702 bands were observed among the 6 primers out of which 676 ones (96%) were polymorphic.

The highest and the lowest number of bands were associated with the primers 1254 (155 bands) and CRA-23 (85 bands), respectively. The mean number of bands produced by each primer were 117 ones. A total number of 111 amplified sites were observed out of which 110 ones were of the polymorphism type. Accordingly, the mean number of amplified sites created by each primer were18.33. The mean number of bands for each specimen was 4.45, such that the highest and the lowest number of bands belonged to primers 1254 (5.96 bands) and CRA-23 (3.26 bands) (Table 1).

The PIC index varied between 15.9% and 34.4% with the maximum value of 34.4% associated with the forward primer 1254, and the minimum value of 15.9% belonging to the forward primer P8. Moreover, the mean value of the index was 25%. The MI was another index investigated in this study; the index had a range between 1.4 (10.54) and 6.53 (53.23), in which the maximum and minimum values belonged to primers OPA-02 and 1254, respectively. Table 3 represents the results of RAPD.

The Results of Experiments Conducted Using the RAPD Primers

According to the Jaccard, coefficient ranged from 0.154 to 0.937. The lowest level of genetic similarity was observed between P. L1 and G. L24 and highest level was observed between B. L9 and B. L7. The cophenetic correlation calculated based on the Jaccard coefficient was equal to 0.93.

Considering the ability of RAPD technique to discriminate isolates with a nearly similar structure, the whole genome was investigated by the technique. In this step, as previously explained, the presence and absence of the band were scored as 1 and 0, respectively (Figure 1). The dendrograms resulted from RAPD data were categorized into 4 main groups at the similarity level of 20%. The first group, which was the largest one, contained isolates from all four populations, suggesting that these specimens had not been geographically separated in an accurate manner. There was only one isolate from the G population in the fourth group, which could be considered as an out group (Figure 2).

The results obtained from the principle component analysis confirmed the grouping performed based on the Jaccard coefficient. Principle coordinates analysis (PCoA) demonstrated that the first and second components

Table 3. Results of RAPD Primers

Primer	NTB	NPB	PPB (%)	SR	PIC	MI
OPA-02	86	60	69.76	700-3000	0.25	1.4
CRA 23	85	85	100	400-2800	0.23	3.97
OPL-02	125	125	100	300-3000	0.25	6.25
P_8	102	102	100	200-1500	0.16	2.7
$M_{13}V$	149	149	100	400-3000	0.26	6.31
1254	155	155	100	250-2200	0.34	6.53
Mean	117	112.6	94.96	-	0.25	4.52
Total	702	676	-	-	-	-

Abbreviations: Number of total bands (NTB), number of polymorphic bands (NPB), percentage of polymorphic fragment (PPB), polymorphism information content (PIC), marker index and size range of amplified fragments (SR).

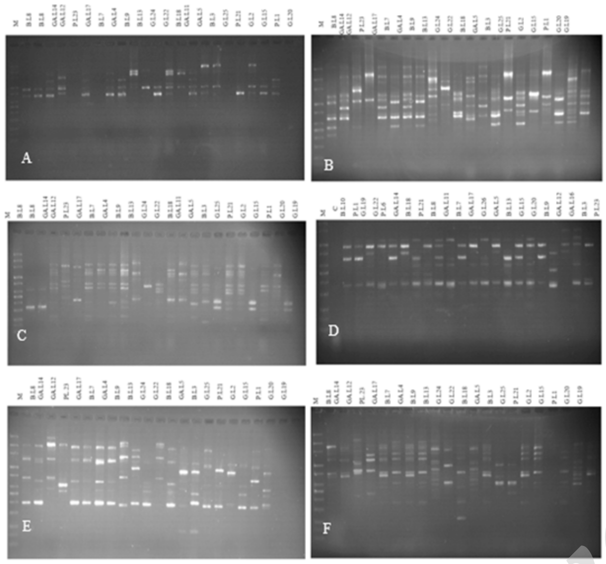

Figure 1. Electrophoretic Patterns of Amplified DNA Segments on the 1.5% Agarose Gel With 100 Base-pair Markers (A: Lactobacilli isolated by the OPA-01 primer, B: Lactobacilli isolated by the M13V primer,C: Lactobacilli isolated by the OPA-02 primer, D: Lactobacilli isolated by the P8 primer,E: Lactobacilli isolated by the CRA23 primer, F: Lactobacilli isolated by the 1254 primer).

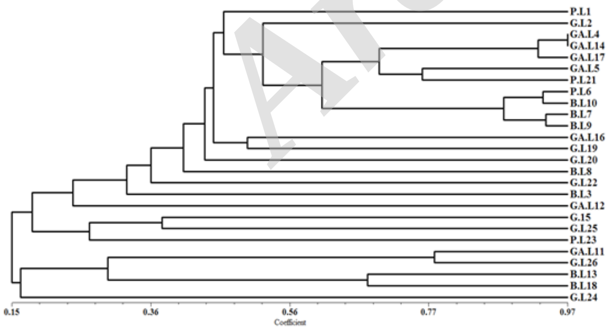

Figure 2. UPGMA Clustering of Isolated Based on Jaccard Similarity Coefficient Calculated From RAPD Markers.

explained 30.59% and 22.48% of variances, i.e. 53.07% of variances in total (Figure 3).

The mean number of observed alleles (Na), the number of effective alleles (Ne), Nei's genetic diversity (h), and Shannon's information index (I) are presented in Table 4. According to this table, the range of genetic diversity among sub-populations, based on the Nei index, varied from 0.242 for P to 0.331 for G.

The genetic similarity among the four populations varied from 62.2% to 73.6%. The lowest genetic distance (i.e. the highest genetic similarity) was observed between P3 and P4 (30%) and the highest genetic distance (i.e. the lowest genetic similarity) was observed between P3 and P4 (Figure 4). According to the cluster analysis performed using UPGMA method, P3 and P4 populations located in the same group, indicating that their genetic similarity was higher compared with that of other populations (Figure 4).

Analysis of Molecular Variance

The results of AMOVA conducted on the four populations are presented in Table 5. It can be seen from this table that intra-population diversity was higher than inter-population diversity. The variance of specimens within the populations accounted for 94% of the total variance, while the inter-population variance accounted for only 6% of the total variance.

Discussion

Studies have demonstrated that traditional dairy products are enriched sources of probiotic bacteria, which may

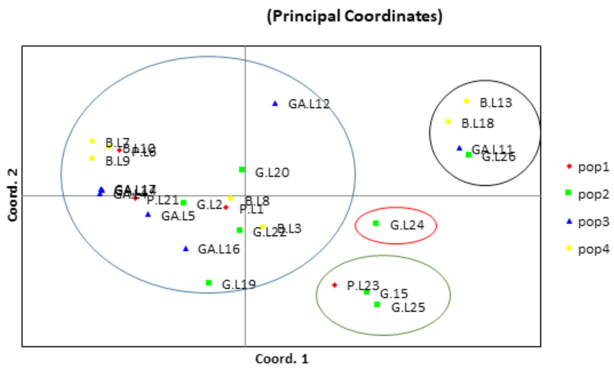

Figure 3. Plot 2 Dimension for PCoA Analysis Based on Jackard Similarity Matrix Calculated From RAPD Data.

Table 4. Genetic Diversity Data and Differentiation Parameters for 4 Natural Populations

Population	N	Na	Ne	H	I	PL	%P
Pashblagh (P)	4	0.865	1.286	0.242	0.166	51	40.54
Qaletapeh (G)	8	1.432	1.386	0.360	0.236	81	70.26
Qaderabad (GA)	7	1.27	1.317	0.313	0.202	71	63.06
Bathche (B)	7	1.198	1.385	0.331	0.224	68	53.56
Mean	-	1.191	1.344	0.311	0.207	-	-
Total	26	-	-	-	-	-	58.11

Abbreviations: Sample size (N), number of observed alleles (Na), number of effective alleles (Ne), Nei's gene diversity (H), Shannon's information index (I), number of polymorphic loci (PL), percentage of polymorphic loci (PPL).

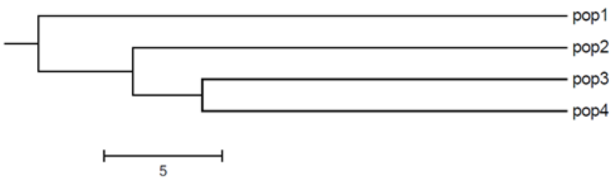

Figure 4. UPGMA Dendrogram of 4 Populations Based on Nei's Genetic Distance by POPGene Software.

not be found in industrial counterparts. Accordingly, such bacteria should be detected, classified, and then added to industrial dairies (14). The use of molecular markers, such as RAPD, is very helpful in discriminating the genetic diversity of bacterial species. In this regard, RAPD-PCR technique is an accurate and simple-to-administer technique with many advantages such as use of a small amount of DNA, no need to use specific primers, detectable on the agarose gel, low cost, and being fast (28,29). Many researchers have utilized the marker to investigate the gene diversity of various genotypes of bacteria, including *Lactobacillus*. Moreover, it has been well accepted that initial screening and isolate selection, based on biochemical and phenotype methods, is not accurate enough because these methods are unable to discriminate bacteria isolates which are readily distinguishable using molecular methods. Interestingly, the results of biochemical and molecular methods are incompatible (30,31). The same results were reported by Abriouel et al (32), a study conducted on the diversity of microorganisms presented in the Alberquilla cheese produced from a mixture of goat and sheep milk. The superiority of RAPD-PCR method over other methods have been demonstrated by many studies, such as Perez Pulido and colleagues' (33) conducted on caper, a fermented fruit, and Svec and colleagues' (34) that reported RAPD-PCR was a more accurate technique in isolating *Lactobacillus* species (including *fermentum*, *rhamnosus* and *casei* subspecies paracasei, as well as gasser and plantarum) from children than did ribotyping. Likewise, the same conclusion was made by Kwon (35) and Latifi et al (30). Kwon investigated the genetic relationship of 6 *Lactobacillus* strains and five isolates were extracted from fermented milk using the PARD-PCR method. A total number of 42 primers were utilized in the present study and the results were analyzed using NTSYS software. All *Lactobacillus* isolates were categorized into 3 separate groups (35).

In the current study, all primers except for the OPA-02 presented the maximum percentage of polymorphism (100%) indicating the high efficiency of such primers in discriminating *Lactobacillus* isolates. Consequently, these markers are recommended to be used in similar studies. Moreover, the genetic diversity of *Lactobacillus* isolates from specimens collected from four different populations were investigated using 2 markers of RAPD and PCR-RFLP; the dendrograms obtained from both markers were unable to provide a satisfactory geographical discrimination, which can be due to the closeness of these geographical areas to each other. The range of genetic similarity in terms of the Jaccard's similarity coefficient of the RAPD marker was between 0.154 and 0.937. As a result, the isolates with a similarity coefficient of 56% were divided into 16 groups.

According to the dendrogram resulted from Jaccard coefficient and UPGMA method, the highest level of genetic similarity was observed between B.L7 and B.L9, postulating that they were 2 different strains from the

Table 5. Result of AMOVA for 4 Populations of Lactobacillus by RAPD Marker

Source (S.O.V)	*df*	SS	MS	POV	EV
Among populations	3	58.533	19.51	6%	0.87
Within populations	22	306.929	13.95	94%	13.95
Total	25	365.462	-	100%	14.82

Abbreviations: df, degrees of freedom; SS, sum squares; MS, mean squares; POV, Popoviciu's inequality on variances; EV, estimated variances.

same genotype, because they were taken from the source. The G.L15, G.L25, and G.l23 isolates, which were located in the same group, were all in the samples taken from the cow. The third group, which was the largest one, contained several isolates associated with GA, P, and B populations, indicating that these populations had not correctly been separated in terms of geographical location.

Conclusion

The geographical distance between the populations was nearly low and they were discriminated appropriately in this regard. These populations had the same climate and this fact could be contributed to the low variance found among populations. The low sample size can serve as another reason for this. The P1 population had both the highest and the lowest genetic similarity with other 3 populations, suggesting that this population had a higher distance from other geographical areas of which the other samples were taken.

Conflict of Interests

The authors declared no conflicts of interest.

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