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

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 immune systems. Moreover, RAPD-PCR is an appropriate method for detection and classification of lactobacillus.

Keywords: Probiotic, Lactobacillus, RAPD-PCR
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 37°C 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 37°C 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).

\[
PIC_i = 2.f_i \cdot (1-f_i) \quad \text{Eq. 1; and MI} = PIC_i \cdot N \cdot \beta \quad \text{Eq. 2}
\]

Where, N denotes the total number of bonds for each primer and \( \beta \) 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          | CGGATCCCCCA      | 10                | (36)       |
| OPL-02          | TGGCCGTCAC       | 10                | (36)       |
| Ps              | CGTACAGGCT       | 10                | (36)       |
| M1V             | GTTTTCCAGTACGAC  | 17                | (36)       |
| 1254            | CCGCAGGCAA       | 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).

**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 were 18.33. 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.

### 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)        |
| GAL4   | Bukan - Qaderabad (Cheese)       |
| GAL5   | Bukan - Qaderabad (Cheese)       |
| GAL11  | Bukan - Qaderabad (Yoghurt)      |
| GAL12  | Bukan - Qaderabad (Yoghurt)      |
| GAL14  | Bukan - Qaderabad (Lurk)         |
| GAL16  | Bukan - Qaderabad (Lurk)         |
| GAL17  | Bukan - Qaderabad (Lurk)         |
| B.L9   | Bukan - Bathche (Yoghurt)        |
| B.L10  | Bukan - Bathche (Yoghurt)        |

**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...
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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 3.

| 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|
| P8       | 102 | 102 | 100     | 200-1500 | 0.16 | 2.7 |
| M13V     | 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 | -       | -    | -   | -  |

Table 3. Results of RAPD Primers

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).

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
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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 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 (P).

| 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   | 58.333 | 19.51 | 6%  | 0.67 |
| 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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