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RESEARCH ARTICLE

Isolation, identification and investigation, of genetic diversity of *Lactobacillus* bacteria strains in traditional dairy products of Lorestan province (Iran)

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Abstract: Traditional dairy products contain a diverse set of bacteria that produce useful biological compounds called bacteriocins. Lorestan province is rich in natural flora due to its special ecosystem and traditional dairy products. In this regard, to isolate, identify and investigate the genetic diversity of *Lactobacillus* bacteria found in dairy products, 10 samples from each of the traditional yogurt, dough, and cheese products of Lorestan province were collected and evaluated. Primary bacteria were isolated using special culture medium (MRS) and various biochemical tests including gram test, catalase test and antibiogram test. In the next step, to confirm and identify the isolated bacteria, 16SrRNA gene amplification and sequencing were used. After identifying the bacteria, the genetic diversity between the isolated bacterial strains was evaluated using REP-PCR and BOX-PCR genetic markers. The antibiogram test showed that all isolated bacterial strains were resistant to vancomycin and kanamycin and sensitive to gentamicin. After the amplification of 1500 base pairs of 16SrRNA gene and its evaluation in databases, it was found that the strain isolated from the dairy products yogurt, dough and cheese were the most similar to *Lactobacillus casei*, *Limosilactobacillus fermentum* and *Lactobacillus helveticus* bacteria respectively. The grouping of the studied bacterial strains based on the similarity matrix of the combination of two markers showed that the studied bacteria formed 7 groups at a similarity coefficient of 45%, and the bacteria isolated from Noorabad local yogurt formed only one group. The results of the present study showed the high potential of traditional dairy products of Lorestan province in terms of having probiotic strains with high genetic diversity, which can be considered as suitable biological preservatives and used to produce of various dairy products commercially.

Keywords: Bacteriocin, dairy, genetic marker, *Lactobacillus*, Lorestan

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Introduction

In recent years, the reduction of food losses due to corruption in the stages of food production has become the focus a lot of important and practical research (Evivie et al. 2020). With the discovery of the positive effects of different species of bacteria on human health in the early 20th century, the first study steps were taken towards research in the field of probiotics. Today, probiotics are classified as live microorganisms that, when consumed in sufficient quantities, can provide many health benefits, and improve the immune system (Meyer-Torpa et al. 2021). The use of beneficial microorganisms in the food and pharmaceutical industries has a long history. Probiotics prevent the growth of spoilage bacteria and pathogens during food storage. Therefore, probiotics are an important way to preserve food and prevent the spread of pathogens through food. Probiotics also have very strong antioxidant properties that prevent food oxidation during food storage (Biolcati et al. 2022).

One of the most important things that must be considered to produce a probiotic product is the ability to transport and distribute, store and manage without losing its viability (Jarocki et al. 2020). Several physical and chemical factors, including oxidation, humidity changes, osmotic pressure, and temperature, affect the viability of probiotic microorganisms. In addition, the conditions of the human digestive system are not suitable for many probiotic species (Derrien and Hylckama Vlieg. 2015).

Lactic acid bacteria (LABs) are of high economic importance and play an important role in the fermentation process of traditional dairy products. Their metabolic properties help to develop the desirable characteristics of food products and allow the nutritional value of raw materials to be maintained and often increased (Linares et al. 2017). Many species of *Lactobacillus* bacteria are in the group of probiotics. These bacteria are tolerant to many environmental changes during manufacturing and processing, storage and transportation, as well as digestive tract conditions. Therefore, they can be a suitable source for the development of probiotic products (Toole et al. 2017).

There are a wide range of laboratory techniques for the identification of lactic acid bacteria, and they can be evaluated based on morphological, biochemical, physiological, and genetic characteristics using different methods. Molecular genetic techniques are the main tools for the analysis of microorganisms and are expanding rapidly. One of the most widely used techniques for bacterial identification is the use of specific primers and amplification of all or part of the 16SrDNA gene sequence (Ferone et al. 2020).

Various DNA fingerprinting techniques exist to access the genetic diversity of different microorganisms, and the use of primers for specific genomic regions provide specific diversity patterns that are very efficient for isolating and grouping microorganisms. Repetitive exogenous palindromic sequence amplification (REP) and BOX are among the fingerprinting techniques developed for genetic analysis in prokaryotes (Borba et al. 2020). Analysis using primers of these regions with repetitive elements creates specific diversity patterns for each bacterium, which can be a suitable tool for distinguishing and evaluating genetic diversity between bacterial strains (Korvin et al. 2014).

LABs can inhibit food pathogens and increase the life of food (Fidana et al. 2022), therefore, adding these bacteria as food supplements is of great interest (Munekata et al. 2021). The natural flora of dairy products is complex and includes many strains of LABs. Since these dairy products containing these bacteria have better and unique flavors compared to products made from pasteurized milk (Biolcati et al. 2022). Due to the high importance of lactic acid bacteria in the food industry, identification, and evaluation of new strains of this group of bacteria can be a very important step in improving the production of dairy products. Based on this, the present study was carried out to isolate, identify and investigate the genetic diversity of lactic acid bacteria in traditional dairy products of western Iran, including different geographical areas of Lorestan province.

Materials and methods

Bacterial strains and growth conditions

A total of 30 acid lactic bacteria isolated from traditional yogurt, dough, and cheese. Samples were collected from different regions of Lorestan province with different geographical distances and weather conditions. After sampling, the samples were transferred to the laboratory in sterile containers on ice and kept in a refrigerator at 4°C for further processing. Isolates were inoculated in selective media MRS broth and incubated under anaerobic conditions (10% CO_2) at 37ÚC for 48 hours. The bacterial isolates examined, and their sources are listed in Table 1.

Morphological and biochemical characterization

For each strain, a liquid culture was grown from a single colony. All isolates were identified according to their morphological and biochemical characteristics. To determine the type of bacteria, gram test and observation under the microscope were used. For catalase test, a drop of 3% hydrogen peroxide was added to a fresh culture on a sterile glass slide and mixed well. Producing bubble or froth, indicated catalase-positive and no bubble or froth indicated catalase negative. After determining the type of bacteria, the antibiogram test was used to determine the pattern of sensitivity and resistance of bacterial strains. For the antibiogram test, 6 commercial antibiotics (tetracycline, vancomycin, amikacin, ampicillin, kanamycin, and gentamicin) were used by the disk diffusion method.

DNA isolation

Total DNA was extracted from a culture inoculated with a single colony. Bacteria cells were grown in 1.5 ml MRS broth for 24 h at 37° C temperature and genomic DNA extractions of the isolates were extracted using the Bacterial DNA Isolation Kit (Denazist, Iran) and the quality and quantity of DNA were measured to determine acceptable purity using spectrophotometry (Bio-Rad SmartSpec 3000 UV/Vis Spectrophotometer, USA) and electrophoresis on 1 % agarose gel, in 1X TAE buffer.

Oligonucleotide primers

Repetitive sequence based on polymerase chain reaction (REP-PCR) fingerprinting with BOX-PCR and REP-PCR was conducted to obtain the genomic fingerprinting of all bacterial isolates. The oligonucleotide primers used in present study are as listed in

Table 1: Bacteria isolated used in this study

Isolated code	Source	Isolated code	Source
B1	Yoghurt	B16	Dough
B2	Yoghurt	B17	Dough
BC	Yoghurt	B18	Dough
B4	Yoghurt	B19	Dough
B5	Yoghurt	B20	Dough
B6	Yoghurt	B21	Cheese
B7	Yoghurt	B22	Cheese
B8	Yoghurt	B23	Cheese
B9	Yoghurt	B24	Cheese
B10	Yoghurt	B25	Cheese
B11	Dough	B26	Cheese
B12	Dough	B27	Cheese
B13	Dough	B28	Cheese
B14	Dough	B29	Cheese
B15	Dough	B30	Cheese

Table 2.

16SrDNA Amplification and Sequencing

Fragments of the 16SrDNA genes were amplified using the universal primers (Table 2). PCR-mediated amplification was carried out in a gradient MyCycler™ thermal cycler system (Bio Rad, USA). The amplification conditions were performed in 25 µl volumes containing 50 ng of template DNA, 10X PCR reaction buffer containing 20 mM $MgCl_2$, 10 pmol each of the primers, 2.5 mM of the dNTPs mixture and 1 U of Sinaclon *Taq* DNA polymerase (Sinaclon, Iran).

The 16SrDNA-PCR consisted of an initial denaturation step at 95°C for 5 minutes, which was followed by 40 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 4 minutes, and a final extension at 72°C for 10 minutes.

The PCR products were checked for correct amplification by electrophoresis on 1.5% agarose gel under UV illumination. PCR products were purified and sequenced with the same primers used in a PCR (Denazist, Iran). Identification and similarity search of the 16SrDNA sequences was carried out using the BLASTN program at the NCBI database (*http.www.ncbi.nlm.nih.gov*) for identification of bacterial isolates at species levels. Phylogenetic analysis was performed sequence alignment using ClustalW and phylogenetic trees were constructed using the neighbor-joining method of MEGA6 program.

BOX and REP-PCR conditions

PCR amplification was performed in 25 µl volumes containing 50 ng of template DNA, 10X PCR reaction buffer containing 20 mM $MgCl_2$, 10 pmol/µl each of the primers, 2.5 mM of the dNTPs mixture, and 1 U of Sinaclon *Taq* DNA polymerase (Sinaclon, Iran). PCR amplification was performed in a gradient MyCycler™ thermal cycler system (Bio Rad, USA).

The BOX-PCR consisted of an initial denaturation step at 95°C for 5 minutes, which was followed by 40 cycles of 94°C for 1 minute, 61°C for 1 minute, 72°C for 4 minutes, and final extension at 72°C for 10 minutes. For REP-PCR, the conditions were 95°C for 5 minutes, which was followed by 40 cycles of 94°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute, and a final extension for 10 minutes at 72°C.

Amplified PCR products were evaluated by electrophoresis on 1.5 % agarose gel, in 1X TAE buffer and visualized under UV light by safe staining and photographed using Gel documentation system (Bio-Rad, USA). To determine the size of the amplified fragment, 100bp DNA ladder (Sinaclon, Iran), was used as size standard molecular marker.

Calculation of genetic diversity and cluster analysis

The presence (1) and absence (0) of REP-PCR and BOX-PCR products were recorded and assembled in a data matrix for each isolate. Analysis of the binary scores was performed using NTSYS-pc version 2.1 software (Rohlf. 2002). A similarity matrix 30 bacteria was calculated using of the simple matching coefficient. The clustering and draw of dendrogram was based on unweighted pair group method with arithmetic averages (UPGMA).

Results and Discussion

Gram, catalase and antibiogram test

Gram test results showed that most of the grown bacteria were Gram-positive bacteria (purple color) and bacilli and negative catalase. However, in some cases, Gram-positive cocci-shaped bacteria were also observed. After isolation of Gram-positive bacteria grown on MRS culture medium, antibiogram test and disc diffusion technique were used to group and more accurately identify the isolated bacteria. Based on the results, all the studied bacteria were resistant to vancomycin and kanamycin and sensitive to gentamicin. Based on resistance and sensitivity to three antibiotics, tetracycline, amikacin and ampicillin, the isolated bacteria had different patterns.

Bacteria isolated from yogurt showed resistance to tetracycline, amikacin and ampicillin antibiotics, while bacteria isolated from dough and cheese were sensitive to tetracycline antibiotics. Bacteria isolated from dough and cheese were generally sensitive to the antibiotic ampicillin, while bacteria isolated from yogurt were relatively resistant to the antibiotic ampicillin. In general, based on the level of sensitivity and resistance to the studied antibiotics, the isolated bacteria showed significant differences.

Table 2: Primers and conditions used in the PCR experiments

Name of primer	Sequence (5'-3')	Annealing Temperature
REP	5′-GTGGTGGTGGTGGTG-3′	56
BOX	5′-CTACGGCAAGGCGACGCT-3′	61
16SrDNA-F	5′-AGAGTTTGATCCTGGCTCAG-3′	60
16SrDNA-R	5′-AAGGTTACCTCACCGACTTC-3′	

Amplification and sequencing of 16SrDNA gene

The results of amplification of the 1500 bp fragment of 16SrDNA gene showed that the specific primers were able to amplify this gene in all the studied bacterial strains (Figure 1). After the successful amplification of 16SrDNA gene, the PCR product was sequenced. The target sequence was aligned against other genetic information of Lactobacillus family bacteria using BLAST tool in NCBI database. The results showed that the isolated bacteria belonged to different species of *Lactobacillus* genus.

To determine the phylogenetic relationships between the isolated bacterial strains, a multiple alignment was performed between the 16SrDNA gene sequences of three bacteria isolated from the dairy products of yogurt, dough, and cheese, and finally a phylogenetic tree was drawn. The results showed that the bacteria isolated from yogurt and cheese were placed in the same group with 87% similarity, and the bacteria isolated from dough formed a common group with these two bacteria at a further distance (Figure 2).

Phylogenetic tree and determination of bacterial strain

Based on the results of BLASTN and determining the similarity between the nucleotide sequences of the 16SrDNA gene, 22 bacterial strains from different species of *Lactobacillus* genus were selected based on the most genetic similarity with the isolated bacteria, and a phylogenetic tree was drawn based on the results of multiple alignment in MEGA software and ClustalW tool. The results showed that the bacterial strain isolated from yogurt (Lorestan-A) was 99% similar to *L.helveticus* bacteria and 95% similar to *Lactobacillus casei* bacteria. The bacterial strain isolated from the dough sample (Lorestan-B) was similar to *Limosilactobacillus fermentum* bacteria with 89% similarity, and the bacterial strain isolated from the cheese sample (Lorestan-C) belonged to *L.helveticus* species with 98% similarity (Figure 3).

Genetic diversity of the studied bacterial strains

The results of PCR product electrophoresis for two REP-PCR and BOX-PCR markers showed that the REP-PCR marker was polymorphic between bacterial strains with the amplification of 15 fragments (bands) and about 91% polymorphism. The BOX-PCR marker with 11 bands and 87% polymorphism showed differences between the studied bacterial strains (Figure 4).

The grouping of bacterial strains based on the combination of information from two markers showed that the studied bacteria formed 7 groups with a 45% similarity coefficient. Genotype number 3 (B3 isolated from yogurt sample) formed a group separately (Figure 5).

The results showed that the grouping was largely consistent with the type of dairy product. So that yogurt, dough and cheese samples were separately placed in similar groups. The grouping results obtained were not consistent with the geographical distances and the bacterial strains isolated from dairy products of similar geographical areas were placed in different groups (Figure 5). In order to check the accuracy of grouping, principal coordinate analysis (PCOA) and grouping based on two principal components (two-dimensional) and three principal components (three-dimensional) were performed. The obtained results confirmed the grouping and showed that the bacterial strains that were isolated from different dairy products were located at a

Fig. 1 Amplification of 1500bp of 16SrDNA gene using PCR technique (The numbers 1 to 20 indicate the number of the bacterial strain and M indicates the marker with a standard size of 1Kb)

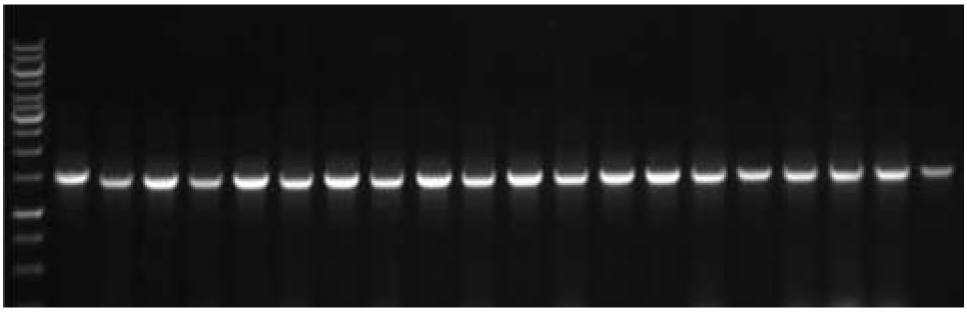
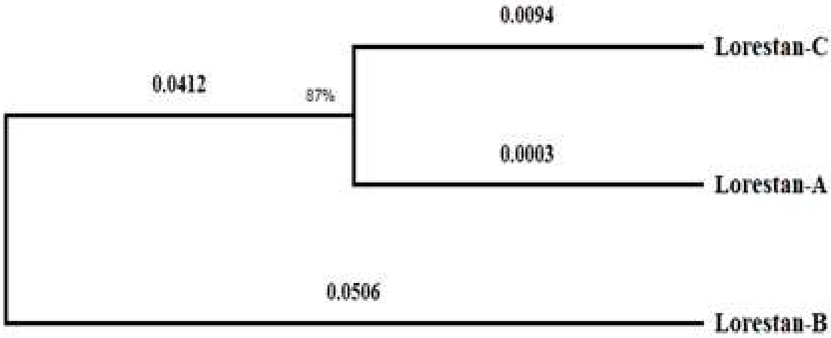


Fig. 2 Phylogenetic tree of selected bacterial strains based on the nucleotide sequence of 16SrDNA gene (Lorestan-A strain of bacteria isolated from yogurt, Lorestan-B strain of bacteria isolated from dough and Lorestan-C strain of bacteria isolated from cheese)



further distance from each other (Figure 6).

Identifying and collecting native strains of probiotic bacteria in traditional products, in addition to maintaining and organizing germplasm, can be used in the production of industrial products using useful and high-efficiency strains. Local dairy products and traditional fermented foods are rich in probiotics and have beneficial and health-promoting properties due to the presence of probiotic bacteria, including lactic acid bacteria (Callon et al. 2004). Probiotic microorganisms include a diverse group of bacteria and fungi that exist symbiotically with other organisms and play a very decisive role in creating the natural balance of ecosystems (Rojek et al. 2022).

Due to the rapid development of the probiotic industry, there is an urgent need to identify new probiotics. To obtain new lactic acid bacteria with high probiotic potential, it is very important to investigate and isolate bacterial species and strains in dairy products (Zhanget al. 2022).

The results of the present study showed that the use of morphological and biochemical techniques such as gram test and catalase test can be very effective in primary separation and reducing the number of examined samples. Lactic acid bacteria in traditional dairy products collected from Lorestan province were all gram-positive and catalase-negative, which was consistent with the results of previous studies. Borga et al. (2017) in their

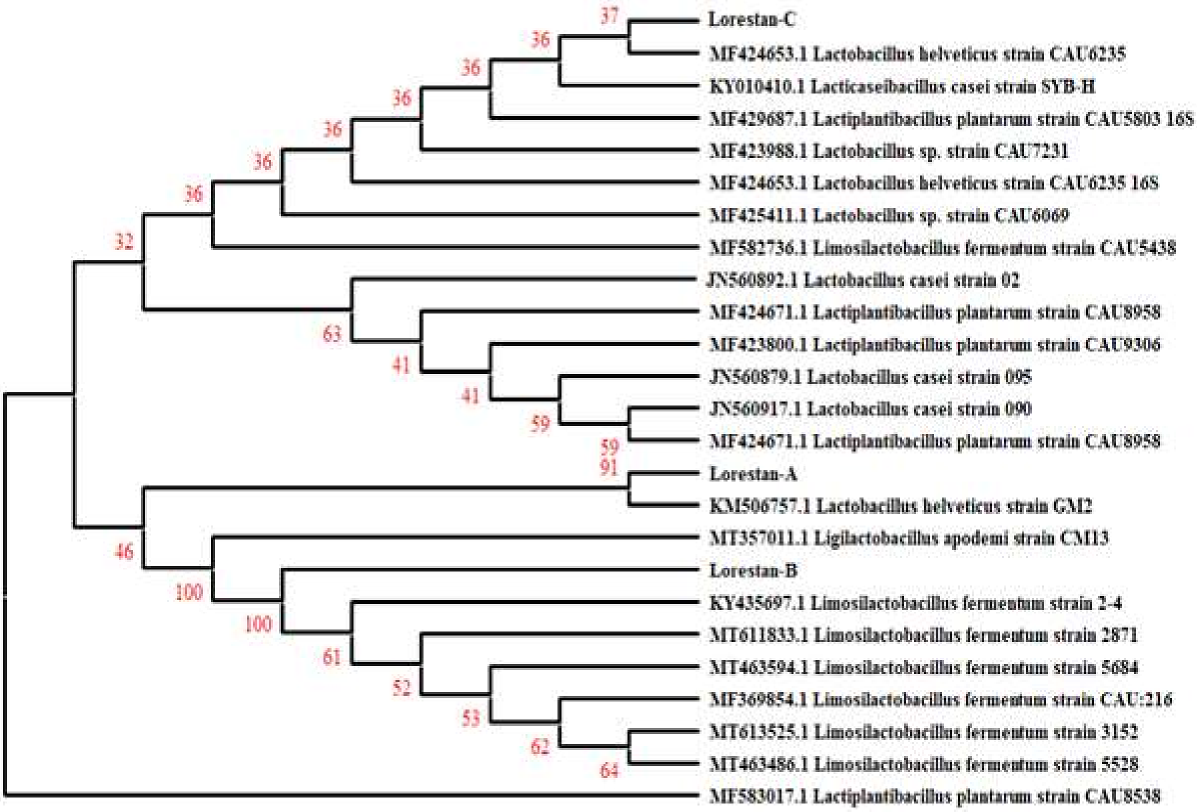


Fig. 3 Phylogenetic tree of selected bacterial strains with different species of *Lactobacillus* genus (Lorestan-A strain of bacteria isolated from yogurt, Lorestan-B strain of bacteria isolated from buttermilk and Lorestan-C strain of bacteria isolated from cheese)

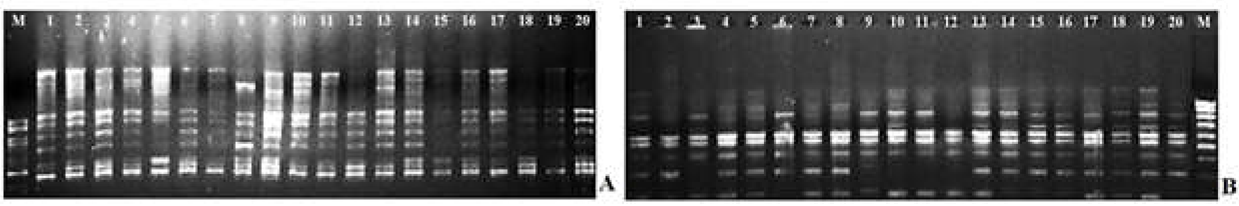


Fig. 4 The results of electrophoresis and amplification of fragments using the REP-PCR (A) and BOX-PCR (B) markers in bacterial strains isolated from the studied dairy products. (Number 1 to 20 include 20 bacteria strains and M represents a marker with a standard size of 100bp)

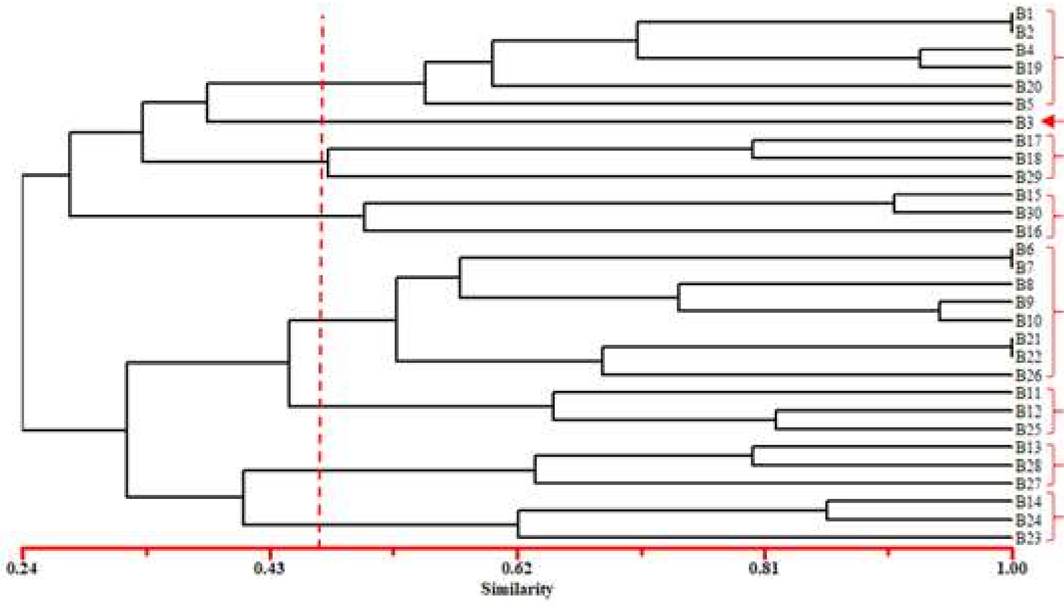


Fig. 5 Grouping of studied bacterial strains using the combination of REP-PCR and BOX-PCR information

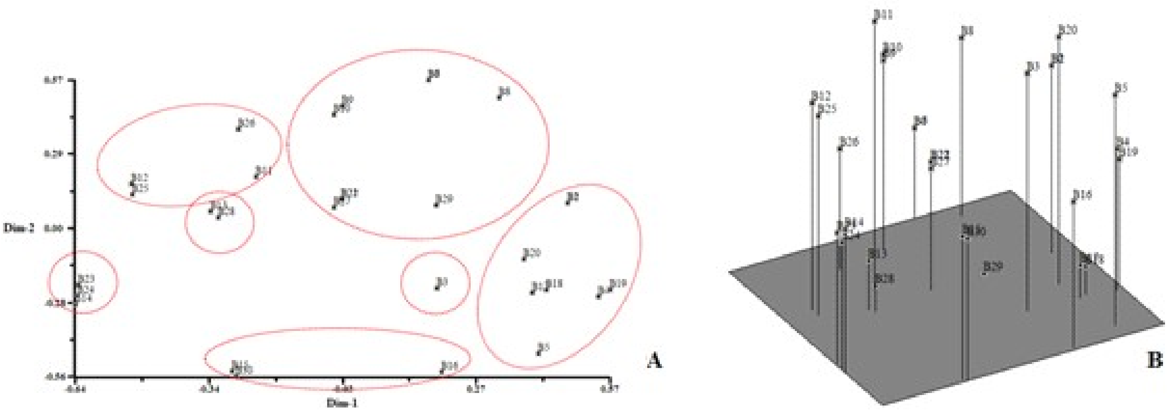


Fig. 6 Grouping of studied bacteria using two-dimensional (A) and three-dimensional (B) graphs drawn based on the PCOA test

research isolated 10 probiotic lactic acid bacteria including *L. acidophilus*, *L. brevis*, *Bifidobacterium,* and *Streptococcus thermophilus* from traditional yogurt samples. In their study, based on morphological, physiological, and biochemical tests, all strains was Gram-positive and catalase negative. In a research, Montazeri et al. (2020) were able to examine 60 strains from a total of 12 raw milk samples with morphological studies and gram test. A total of 36 catalase-negative and Gram-positive strains were found, including *L. gasseri* and *L. galottix*.

In the present study, the antibiogram technique and determining the pattern of sensitivity and resistance of the isolated bacteria showed a suitable grouping and the obtained pattern was like the results of previous studies. Although some observed differences can be attributed to the changes caused by the test method. In a similar study, Zhang et al. (2018) reported different sensitivity and resistance patterns of lactic acid bacteria in dairy products. In their study, 11 different antibiotics were used to group the isolated bacteria. In general, it was found that the use of antibiogram test can be a suitable tool to check the selection of different bacteria.

In another similar study conducted by Junior et al. (2015) on the isolation and identification of lactic acid bacteria, it was found that most of the studied bacterial strains are sensitive to chloramphenicol. They stated that the pattern of resistance and sensitivity depends on various characteristics of bacteria, including the type of bacteria and the presence of resistance and sensitivity genes in the bacterial cell.

The results of the present study showed that all the isolated bacteria were sensitive to the antibiotic gentamicin. Although these results do not confirm natural resistance to gentamicin in all bacteria present in traditional crops, this antibiotic can be used for other similar studies. All isolated strains showed resistance to vancomycin and kanamycin, and about 60% of the samples were resistant to amikacin antibiotic. Tulumoglu et al. (2013) found that 90% of *Lactobacillus* strains tested were resistant to gentamicin. These results indicate the weak inhibition of bacteria in traditional products by aminoglycoside antibiotics.

Lactic acid bacteria (LABs) are mainly sensitive to clinical antibiotics such as penicillin, tetracycline, erythromycin, and chloramphenicol, and they have relatively high resistance to streptomycin and ciprofloxacin antibiotics. However, the pattern of resistance and sensitivity of these bacteria is very different at the level of species and strains, and species of the same genus may have different patterns. The antibiotic resistance gene in *Lactobacillus* bacteria is located on the chromosome and is highly conserved (Dzidic et al. 2008).

The results of molecular analysis of the isolated bacterial strains showed that the bacteria isolated from yogurt are similar to *L. helveticus* and *L. casei* bacteria. The bacterial strain isolated from the local dough sample belonged to *L.fermentum* and the bacterial strain isolated from Lorestan traditional cheese also belonged to *L.helveticus*. The results showed that according to the type of dairy product, the bacterial strain in them is also different.

The natural flora of traditional cheeses is complex and includes several strains of lactic acid bacteria, which are very important in cheese processing and creating its smell and taste. Since these cheeses have better and unique flavors compared to pasteurized and industrially pasteurized cheeses, there is more interest in studying the functional and structural diversity of lactic acid bacteria in traditional cheeses. Therefore, traditional cheeses are considered as the best sources of lactic acid bacteria strains useful for the food industry. The favorable properties of these bacteria are very important for use as starter cultures in dairy products (Coelho et al. 2022).

The results of grouping the studied bacterial strains based on REP-PCR and BOX-PCR markers showed that the studied bacteria have a high genetic diversity. In addition, significant genetic diversity was also observed among similar dairy products. The results generally indicated the appropriate efficiency of DNA-based genetic markers in the grouping of lactic acid bacteria of traditional dairy products.

Based on the results of the study by Gevers et al. (2011), the REP-PCR marker is an efficient and effective tool for the isolation of lactobacilli in traditional dairy products. In their study, they showed that this group of markers with high multiplication power of polymorphic fragments can be very efficient in grouping species and strains of lactic acid bacteria.

Physiological and biochemical tests have limitations and similar physiological characteristics are seen among many of isolated bacteria. In addition, the results of these methods are not always accurate. These results are only based on cultivation methods and phenotypic characteristics, and for this reason, it is recommended to use genetic methods along with phenotypic tests to identify the strain. For this purpose, in the present study, REP-PCR and BOX-PCR molecular markers as well as 16SrDNA gene sequencing were used to group and more accurately identify the isolated bacteria. The results of these techniques were able to identify the type of bacteria studied based on the type of species while grouping the isolated bacteria.

Isolation of new *Lactobacillus* bacteria strains and determination of their characteristics allows the introduction of innovative and competitive probiotic products with increasing applications. During isolation, there will be several limitations in the identification and confirmation of bacteria. Considering that a large number of strains isolated from a source are usually subjected to discriminant analysis, the selection of appropriate techniques will usually be influenced by factors such as the duration and cost of the analysis. Nowadays, methods based on DNA molecular markers such as amplification of repetitive sequences (REP-PCR) are different in terms of potential and required time (Jarocki et al. 2020).

Probiotics isolated from the traditional microbiome can be successfully stored in the genetic bank for use in the pharmaceutical and food industries, especially as culture starters in the future. However, these isolated lactic acid bacteria should be further investigated and their other potential probiotic properties, including anti-cancer properties, intestinal disorders, and anti-allergic properties should be evaluated. Also, due to the increasing use of probiotics, nutritional supplements, and therapeutic agents, there should be detailed information about their risks and benefits (Ahmadnejad and Dolatabadi. 2020).

Conclusion

Examining and evaluating the characteristics of native bacteria strains of each region can be the source of new lactic acid bacteria. This work, while preserving microbial and genetic resources, provides useful information for scientific and commercial applications, especially in the field of dairy and traditional industries. It is necessary to establish a regular structure for collecting, preserving and using lactic acid bacteria in traditional dairy products.

The high efficiency of REP-PCR and BOX-PR markers in identifying the genetic diversity of lactic acid bacteria in dairy products was confirmed. These products are known to be rich in different bacteria with significant probiotic potential. The results of the present study showed the high potential of traditional dairy products of Lorestan province in terms of having probiotic strains with high genetic diversity, which can be considered as suitable biological preservatives and used to produce various types of dairy products commercially. These bacteria can be added to different foods and benefit from their effects.

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