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The Isolation and Identification of Dominant Lactic Acid Bacteria by the Sequencing of the 16S rRNA in Traditional Cheese (Khiki) in Semnan, Iran

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ABSTRACT

Background: Identification of the dominant lactic acid bacteria involved in the production of traditional cheese in Semnan could be the initiative to protect national genetic resources and produce industrial cheese with desirable texture and organoleptic characteristics similar to traditional cheeses. The present study aimed to determine the biochemical, physiological, and phenotypic properties of the dominant strains of the lactic acid bacteria (LAB) isolated from the traditional cheese in Semnan, Iran using 16S rRNA gene sequence analysis.

Methods: In total, 14 cheese samples were randomly collected from the northern countryside of Semnan, Iran. The isolated gram-positive and catalase-negative colonies were analyzed in terms of morphology, culture, physiology, biochemical properties, and carbohydrate fermentation pattern. Following that, the LAB isolates were identified based on 16S rRNA gene sequence analysis, and a phylogenetic tree was drawn for the bacterial strains.

Results: In this study, 105 isolates were determined, 58 of which (55.24%) belonged to the genus Lactobacilli, and 47 isolates (44.76%) belonged to the genus Enterococci. According to the biochemical tests and 16S rDNA sequencing, the identified dominant Lactobacillus species included *L. plantarum* (53.6%), *L. paracasei* (32.7%), and *L. casei* (13.7%). In addition, most of the Enterococcus species were *E. faecium* (74.47%) and *E. durans* (25.53%).

Conclusion: According to the results, exploring the microbiological diversity of traditional cheese could contribute to the selection of proper bacterial strains for the manufacturing of products with consistent quality and original taste on an industrial scale.

1. Introduction

Lactic acid bacteria (LAB) comprise a wide range of genetically distinct bacterial genera, which are highly significant to the food industry and are routinely used in various fermented food products, including dairy products, plant products, and meat [1].

LAB are used in food products for the reduction of food spoilage and extending the shelf life of food products. Today, the beneficial health effects of the bacterial genera belonging to LAB, especially *Bifidobacterium* spp. and *Lactobacillus* spp., have resulted in their increased applications for the production of healthy products, which are referred to as probiotics [2].

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There is remarkable commercial interest for specific LAB (e.g., starter strains) for fermentation purposes or as probiotic strains in various food supplements or products [3]. The identification, screening, and isolation of LAB from natural products are among the most effective methods for obtaining appropriate strains for marketing purposes.

Traditional dairy products are produced from milk without industrial starter strains in artisan environments [4]. Large quantities of various dairy products are traditionally produced from ruminant milk across the world. Khiki cheese is a traditional cheese product in Semnan province, Iran. This type of cheese is produced by the local residents from small ruminant milk with no heat treatment of the milk and no use of starter cultures. Khiki cheese normally passes the ripening period in bags made of ruminant skin (known as Khiki) for six months.

Various sensory and diversity properties of the traditional cheese produced from raw milk depend on their microbial flora [5]. In general, commercial strains are not applied in the production of traditional dairies, and these dairy producers mainly rely on the natural LAB found in milk in order to obtain the required lactic acid properties. This approach contributes to the reduction of pH, which in turn increases the extraction of whey from the curd, thereby lowering the moisture content and decreasing the tendency for microbial spoilage [5].

The demand for the selection of microbial strains with functional properties is on the rise for commercial dairy products in order to enhance the safety and quality of the regular fermented foods currently found on the market [6]. The present study aimed to determine the biochemical, physiological, and phenotypic properties of the dominant strains of LAB isolated from the traditional cheese in Semnan, Iran using 16S rRNA gene sequence analysis to identify and classify the isolates more accurately. Out findings could lay the groundwork for the selection of new strains to be used as specific cultures in the production of this traditional cheese on an industrial scale.

2. Materials and Methods

2.1. Cheese Sampling

In total, 14 samples of traditional cheese were randomly collected from the countryside of northern Semnan city, Iran. The samples were collected aseptically and preserved in sterile, lidded containers. Afterwards, the samples were transferred to the laboratory in iceboxes and preserved in a refrigerator until microbiological analysis and LAB isolation.

2.2. Preliminary Identification and Characterization of LAB

The samples were homogenized using 2% w/v sterile sodium citrate (Merck, Darmstadt, Germany), serially diluted, and placed on Man-Rogosa-Sharpe (MRS) agar plates (Merck, Darmstadt, Germany). The inoculated MRS agar plates overlaid with the MRS agar and incubated in an anaerobic jar using the Anaerocult C gas pack (Merck, Darmstadt, Germany) at the temperature of 37 ºC for 48 hours. The isolated colonies with the typical characteristics of LAB were selected from each plate. Afterwards, the LAB colonies were sub-cultured on the MRS medium using the streak plate technique [7]. All the isolates were gram-stained and assessed microscopically using an Olympus BX41 light microscope (Olympus, Tokyo, Japan). In addition, the catalase activity of the isolates was determined by detecting the breakdown of hydrogen peroxide. Only the gram-positive and catalase-negative isolates were considered as LAB [8].

2.3. Biochemical Properties of the Enterococci Isolates

Various tests were performed for the identification of the coccal LAB isolates at the genus level, including gas production from glucose using the Phenol Red Base Broth medium (Merck, Darmstadt, Germany) containing 1% glucose. Growth rate at various temperatures (10 ºC and 45 ºC), growth rate at the sodium chloride concentration of 6.5% and 18%, and growth rate at the pH of 9.6 and 4.4 were applied based on the method proposed by Axelsson (2004) [8].

The strains that were identified as Enterococci were evaluated in terms of the fermentation of some carbohydrates, potassium tellurite reduction, growth in 10% skimmed milk containing 0.1% w/v methylene blue, and ammonia production from arginine on Moeller Decarboxylase Broth (Merck, Darmstadt, Germany) after the addition of Nessler's reagent in accordance with the method proposed by Kandler and Weiss (1986) [9].

Sugar fermentation tests were performed using 96-well microtiter plates on Phenol Red Base Broth (Merck, Darmstadt, Germany) with phenol red considered as the pH indicator. Moreover, carbohydrates were added to the medium in order to obtain the final concentration of 1% w/v, with the exception of salicin at the concentration of 0.5% w/v. Six types of sugar were used at this stage, including arabinose, arbutin, melezitose, melibiose, sorbitol, and sorbose.

2.4. Biochemical Properties of the Lactobacillus Isolates

In this study, gram-positive and catalase-negative rod-shaped isolates were considered as Lactobacillus strains [10]. The Lactobacilli isolates were characterized based on esculin hydrolysis using esculin agar (Merck, Darmstadt, Germany) at the temperature of 35 ºC for 24 hours and their sugar fermentation profiles. At this stage, 21 types of sugar were used, including amygdalin, arabinose, cellobiose, fructose, galactose, glucose, gluconate, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose (Merck, Darmstadt, Germany). Duplicate reactions were prepared for each sugar fermentation experiment. Moreover, carbohydrate utilization was assessed 24, 48, and 72 hours after the incubation of the tested isolates at the temperature of 30oC based on the methods proposed by Kandler and Weiss (1986) [9].

2.5. 16S rRNA Gene Sequence Analysis DNA Extraction

The pure colonies of the isolated strains on the MRS medium, which had been incubated at the temperature of 30 ºC for 48 hours, were used for DNA extraction. Genomic DNA extraction was performed using the Rose method through the alkaline lysis of the bacterial cells [11]. Following that, the extracted genomes were preserved in

freezers at the temperature of -20 ºC until the molecular assay.

2.6. Sequencing of 16S rDNA

The 16s rDNA sequence coding region was amplified using polymerase chain reaction (PCR) amplifications, which were carried out with the volume of 50 microliters using a thermal cycler (Master Cycler Gradient, Eppendorf AG, Hamburg, Germany). The reaction mixture contained PCR buffer (1×), dNTPs (0.2 mM), Taq DNA polymerase (2 U), MgCl2 (1 mM), 15 pM of each primer, DNA template 1 (µl), and adequate distilled water to achieve the volume of 50 microliters.

Universal primers were used for the PCR amplification of almost complete 16s rDNA sequencing of 1,500 bp of the isolated strains, including 27-F (5′-AGAGTTTGATCA/CTGGCTCAG-3′) and 1525-R (5′-AAGGAGGTGA/TTCC AA/GCC-3′). Moreover, each reaction set consisted of a negative control. The PCR thermal cycler was programmed with the initial denaturation for 10 minutes at the temperature of 94 ºC, 35 cycles of denaturation for 90 seconds at the temperature of 94 ºC, primer annealing for 90 seconds at the temperature of 62 ºC, and extension for two minutes at the temperature of 72 ºC, followed by the extension of the incomplete products for 10 minutes at the temperature of 72 ºC.

At the next stage, the PCR products were separated via electrophoresis on 1% agarose gel for one hour in 1× tris-acetate-EDTA (TAE) buffer at the constant voltage of 85 V. In addition, the PCR products were stained by ethidium bromide (1 mg/ml) and visualized under ultraviolet light, followed by the capturing of digital images using a camera (UVP, Inc., Upland, CA). The size of the amplified fragments was determined through comparison with the DNA ladder (1 kb; CinnaGen, Tehran, Iran). The obtained PCR products were excised and eluted from the gel. Moreover, the amplicons were purified using a GF-1 Gel-DNA recovery kit (Vivantis Resources, Shah Alam, Malaysia) and sequenced by both primers on ABI 3700 automated sequencers (Applied Biosystems, Foster City, CA) [12].

2.7. Data Analysis and Species Identification

Homology comparisons were performed using the Basic Local Alignment Search Tool (BLAST) online at the National Centre for Biotechnology Information (NCBI) homepage (http://blast.ncbi .nlm.nih.gov/Blast.cgi). Following that, the sequences were aligned to construct a phylogenetic tree and compare the similarities among the sequences using the neighbor-joining method in the MEGA software version 6.0 [12].

3. Results and Discussion

3.1. Isolation and Identification of the Dominant LAB

In total, 105 isolates were identified based on their morphological, physiological, and biochemical properties. The gram-positive and catalase-negative organisms were considered as LAB and divided into two morphological groups. The microscopic examination of the isolates revealed 48 cocci (44.76%) and 58 rod-shaped bacteria (55.24%). In order to identify the isolated lactic acid cocci at the genus level, some tests were carried out, the results of which are presented in Table 1.

3.2. Characterization of the Isolates

All the rod-shaped isolates that were gram-positive and catalase-negative were presumed to be of the genus Lactobacillus (55.24%). Based on the carbohydrate fermentation profiles and esculin hydrolysis, the detected dominant Lactobacillus species included *L. plantarum*, *L. paracasei*, and *L. casei* (Table 2). According to the obtained results, the mentioned species comprised of 53.6%, 32.7%, and 13.7% of the bacterial flora isolated from the traditional Khiki cheese samples, respectively.

In order to distinguish the isolated lactic acid cocci at the species level, their biochemical properties were investigated (Table 3). According to the information in Table 3, 35 isolates (74.47%) belonged to the *Enterococcus faecium* species, and 12 isolates (25.53%) belonged to the *Enterococcus durans* species.

3.2. Gene Sequences and Phylogenetic Analysis 16S rRNA

The LAB isolated from the traditional cheese samples collected in Semnan region could be more accurately identified at the species level using 16S rRNA gene sequencing. In the present study, the 16S rRNA gene sequences were successfully amplified from the isolates using the PCR. According to the obtained results, the 16S rRNA gene sequences (continuous stretches of approximately 1,500 bp) were successfully amplified using the universal primer 27f/1525r (Figure 1). In addition, the NCBI BLAST program (http://www.ncbi.nlm.nih.gov) was applied for their closest relative/reference strains with the homology of 94-100% .

Phylogenetic tree analysis (Figure 2) was performed to determine the correlations between the representative isolates and known reference strains. The phylogenetic analysis categorized all the isolates into two clusters and five sub-clusters. The first cluster was the *Lactobacillus* (L) group, with the sub-clusters of *L. plantarum*, *L. paracasei*, and *L. casei*. The second cluster was *Enterococcus* (E), with the sub-clusters of *E. faecium* and *E. durans*.

Table1: Preliminary identification of the lactic acid bacteria isolates at genus level Characterization of the isolates

Groups	Physiological tests							
	CO_2 from glucose	Growth at PH=9.6	Growth at PH=4.4	Growth at 18% NaCl	Growth at 6.5% NaCl	Growth at 45C	Growth at 10C	Preliminary identification
Groups 1(58 isolates)	+/-	-	+/-	-	+/-	+/-	+/-	Lactobacillus
Groups 2(47 isolates)	-	+	+	-	+	+	+	Enterococcus

Table 2: Biochemical characteristics of Lactobacillus isolates

Carbohydrates	Group 1 (31 isolates)	Group 2 (19 isolates)	Group 3 (8 isolates)
Manitol	-	+	+
Manose	+	+	+
Galactose	+	+	+
Glucose	-	-	+
Fructose	+	+	+
Xylose	+	-	+
Sucrose	-	+	-
Arbutin	-	+	+
Cellobiose	+	+	+
Lactose	+	+	+
Maltose	+	+	+
Melezitose	-	+	+
Ribose	+	+	+
Sorbitol	-	+	-
Sorbose	-	-	-
Arabinose	-	+	-
Ramnose	-	+	-
Salicin	+	-	+
Trehalose	+	+	+
Melibiose	-	-	-
Gluconate	+	+	+
Raffinose	+	-	-
Amygdalin	+	+	+
Esculin	-	-	+
Identification on the basis of 16s rDNA analysis	*Lactobacillus plantarum*	*Lactobacillus paracasei*	*Lactobacillus casei*

Table 3: Biochemical characteristics of the enterococcus isolates

Physiological tests	Group 1 35 (isolates)	Group 2 12(isolates)
Melibiose	+	-
Arabinose	+	-
Sorbitole	-	-
Melesitose	+	-
Sorbose	-	-
Arbutin	+	+
Potassium Tellurite reduction	-	-
Ammonia from arginine	-	-
Growth in 0.1% methylene blue milk	+	-
Identification on the basis of 16s rDNA analysis	*Enterococcus faecium*	*Enterococcus durans*

LAB are naturally present as native microflora and are widely distributed in raw milk and dairy products with the potential to act as probiotics [13,14]. In the present study, the sampled traditional dairy products were investigated in terms of the presence of LAB using biochemical and molecular methods. The detected dominant Lactobacillus species included *L. plantarum*, *L. paracasei*, and *L. casei*, which comprised of 53.6%, 32.7%, and 13.7% of the bacterial flora isolated from the traditional Khiki cheese samples, respectively.

In the current research, the analysis of 16S rDNA yielded the same results as the biochemical tests. After the specific PCR, the nucleotide sequences were compared with the nucleotide sequences available in the NCBI gene databank, and *L. plantarum* was confirmed as the strain with the highest level of agreement. According to the results of the present study, the *L. plantarum* species was dominantly present (53.6%) in all the samples of traditional Khiki cheese. *L. plantarum* has a long history of natural presence and safe use in various food products. Nevertheless, some reports have denoted the adverse effects of *L. plantarum* on the quality of cheese. For instance, the presence of *L. plantarum* in Emmental cheese has been reported to disturb the metabolism of propionic acid bacteria, leading to the lower quality of cheese due to the changes in its flavor [15]. Furthermore, *L. plantarum* strains seem to be dominant Lactobacilli in Maasai fermented milk and Kopanisti and goat milk cheese [6].

In a study conducted during the ripening of a Dutch cheese, the findings indicated that *L. plantarum* and *L. casei* converted citrate into CO_2, acetate, acetoin, diacetyl, and 2,3-butanediol in the absence of other energy sources for their utilization, so that CO_2 could contribute to the formation of pores in cheese, as well as the other ingredients that were effective in creating the desired flavor [16]. Considering the comprehensive research focused on the mesophilic Lactobacilli isolated from traditional cheese samples, their role in the production of aromatic ingredients and flavor of cheese have been confirmed. For instance, in the studies evaluating Fossa cheese, it has been reported that *L. plantarum*, *L. paracasei*, and *L. casei* bacteria produce peptides and volatile amino acids during dipeptidase, amino peptidase, endopeptidase, and proteinase processes, which result in the desirable flavor of cheese [16, 17].

Recent studies have confirmed that artisan cheeses have diverse and typical microbial population dynamics, which are associated with the local production technology and geographical regions of their production [18]. In the current research, the genera of the LAB isolated from the traditional Khiki cheese samples were Lactobacillus (55.24%) and Enterococcus (44.76%), which is consistent with the findings of Tzanetakis (1995) in a study conducted on white cheese made of raw sheep milk [19]. As mentioned earlier, *L. plantarum*, *E. faecium*, and *E. durans* constituted 47.6%, 12.3%, and 9.8% of the total isolates, respectively. In another study performed on Lighvan cheese (a traditional Iranian cheese), *E. faecium*, *L. lactis*, and *L. plantarum* were reported to be the dominant species isolated from ripened Lighvan cheese samples [20]. Furthermore, the research conducted by Mathara et al. (2004) in Kenya indicated that *L. paracasei*, *L. fermentum*, *L. plantarum*, and *L. acidophilus* were detected in the traditional dairy product samples (Kule naoto) with the pH of less than 4.5 [6].

In another study in this regard, Misaghi et al. (2017) isolated *L. acidophilus*, *L. paracasei*, and *L. fermentum* from a traditional yoghurt, and the effects of these bacteria on the growth, enterotoxin production, and sea gene expression in S. aureus were investigated in co-culture conditions [21]. On the other hand, some researchers have denoted the effects of isolated Lactobacilli from traditional cheese on some pathogens [22-24]. Accordingly, the isolated coccal LAB could belong to the genus Enterococcus (44.76%).

In order to distinguish the isolated lactic acid cocci at the species level in the present study, their biochemical properties were investigated. According to the findings, 35 isolates (74.47%) were *E. faecium*, and 12 isolates (25.53%) were *E. durans*. Thorough consistency was observed between the results of the biochemical tests of the coccal isolates and 16S rDNA analysis, which indicated ≥ 99% DNA homology between the isolates and the identified species registered in the Gene Bank. The Enterococcus genus has a broad distribution in the environment, and the largest number of the bacteria found in dairy products often belongs to this genus. In addition, the highest number of Enterococci in Iran has been reported in goat milk [20].

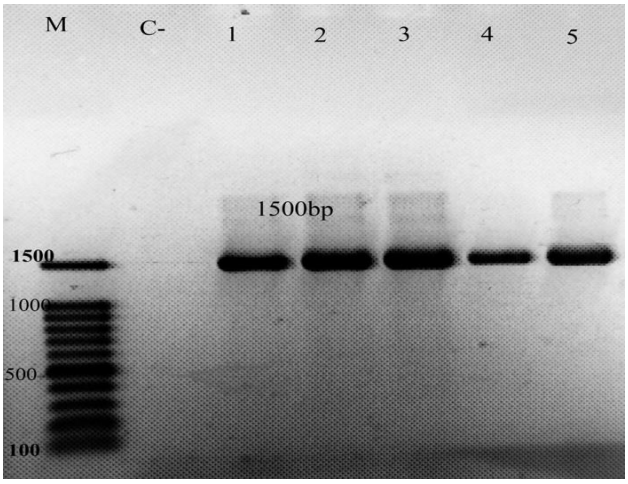

Figure 1: Gel Electrophoresis of PCR Products for Amplification of 16S rRNA Sequence (1500 bp) of Lactic Acid Bacteria (LAB) (Lane M: 100 bp marker, Lane C: negative control, Lanes 1-5: amplified products of LAB isolates)

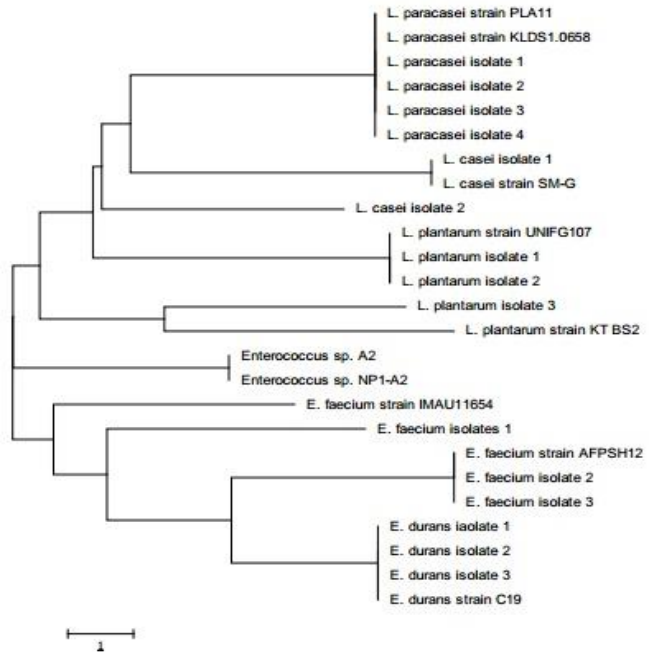

Figure 2: Neighbor-joining Phylogenetic Tree of LAB Isolates Derived from Traditional Cheese of Semnan and Reference Strains Based on Analysis of 16S rRNA Gene Sequences

In the other studies focused on Iranian cheese [25] and other types of cheese (e.g., Serra, Feta, Comte, and Mozzarella) [26], the most dominant bacterial populations have been reported to be of the genus Enterococcus. In the present study, *E. faecium* was observed to be the most dominant species among various Enterococci in traditional Khiki cheese, followed by E. durans. It is also notable that *E. faecium* has been listed as a LAB starter by the International Dairy Federation [27]. According to another research in this regard, *E. durans*, *E. faecalis*, and *E. faecium* were the most frequent bacterial species during the ripening of Montasio cheese [27]. In Semicotto Caprino cheese, the most commonly identified Enterococci have been reported to be *E. faecalis* and *E. faecium*, followed by *E. durans*, *E. hirae*, and *E. gallinarum*. These bacteria are part of the normal intestinal flora and contribute to the ripening and formation of the flavor and aroma of fermented products since they are able to produce proteolytic and sterolytic enzymes for diacetyl production [28].

Some researchers have denoted the positive effects of E. faecalis on the quality of Roquefort cheese [29]. The beneficial effects of Enterococci on the growth of other LAB have also been reported in the literature. Furthermore, the production of bacteriocin (enterocin) by Enterococci has been shown to exert controlling effects on the pathogenic bacteria in cheese, as well as the biogenic amines producing bacteria, such as some strains of Lactobacilli [1]. However, Enterococci have been associated with some adverse effects on health and the quality of cheese. For instance, *E. faecalis* may cause a bitter taste when used as a starter in the production of Gorgonzola cheese [30].

4. Conclusion

According to the results, traditional dairy products have unique microbiological diversity, and the bacterial strains found in these products could be used as a starter culture in the production of commercial cheese with improved quality and health. In general, the presence of *L. plantarum*, *L. paracasei, L. casei, E. faecium*, and *E. durance* in the studied traditional Khiki cheese samples indicated that these strains are compatible with the environment of this type of ripened cheese. Therefore, these microorganisms could be used as starter cultures in the production of this organoleptically popular cheese on the hygienic and industrial scales. It is recommended that further investigations be conducted in this regard in order to assess the probiotic properties of LAB isolates.

Authors' Contributions

M.P., designing the study and writing of the manuscript; M.Kh., and H.S., obtaining the samples and conducted the experimental work; A.J.J., conducting the statistical analyses.

Conflict of Interest

The authors declare that there is no conflict of interest.

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