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Identification of Lactic Acid Bacteria Isolated from Traditional Iranian Lighvan Cheese

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Abstract: Many traditional products such as cheese obtain their flavor intensity from non-starter lactic acid bacteria (NSLAB), which are not part of the normal starter flora but develop in the product, particularly during ripening, therefore it was decided to identify this group of bacteria from Iranian Lighvan cheese, made from raw ewe's milk for the establishing of new functional foods and/or increasing products to cover the demands of the Iranian market. Ten grams of cheese samples (in triplicates) were homogenized in a stomacher and using microbiological analysis for identification of the isolated bacteria. NSLAB formed a large part of the lactic flora at 4 months of ripening. Forty-two typical colonies of lactic acid bacteria (LAB) were selected out of 215 isolates from samples of Lighvan cheese. Isolates were phenotypically characterized by their ability to ferment different carbohydrates. Accordingly they were classified into 3 predominant genera: *Lactobacillus, Enterococcus* and *Pediococcus* (46, 42 and 12%, respectively). All isolates of *Enterococcus* were identified as *E. faecium*. In case of *Lactobacillus* isolates, many similarities to species *L. plantarum* and *L. casei* were spotted. The carbohydrates fermentation patterns for *Pediococcus* isolates were unusual and identification at the species level needs further investigation.

Key words: LAB, NSLAB, *Lactobacillus, Enterococcus, Pediococcus*

INTRODUCTION

Lighvan cheese is a semi-hard traditional Iranian cheese, originally from Lighvan village, East of Tabriz, Iran. It is traditionally made from unpasteurised ewe's or goat's milk, or mixtures of both. Iranian, in general prefer it to other cheeses due to its excellent natural taste and flavor.

The production of all fermented dairy products is based on the use of starter cultures[1,2] such as lactic acid bacteria that initiate rapid acidification of the raw material and can contribute to the microbial safety or offer one or more organoleptic, technological and/or nutritional advantages to improve the quality of product[2-6].

LAB are widely distributed in the nature, they are typically involved in a large number of the spontaneous food fermentations[6,7]. This group is included representatives of the genus *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc*[6]. The wild strains of this group, from biotechnological aspect, are known as bacteriocin producers and probiotics[5,6]. They are Generally Recognized As Safe (GRAS) microorganisms[8] that have a potential use for the establishing of new functional foods[2, 6].

Moreover, many traditional products obtain their flavor intensity from non-starter lactic acid bacteria (NSLAB), which are not part of the normal starter flora but develop in the product, particularly during maturation, as a secondary flora[2, 3, 9-12]. NSLAB are able to transform milk constituents into volatile compounds, which may play a critical role in development of cheese flavor[2,13-17]. The origin of NSLAB is not well known, they may enter the cheese vat either through the milk, or other ingredients used for the cheese making[3,18].

There is an increasing requirement to select microbial strains with functional properties for commercial production and for improvement of quality and safety of existing traditional fermented food products[10,19] although the influence of microbial flora on the sensory properties of many raw milk cheese has not exactly been established[11,15,20].

Today, Lighvan cheese has a large market popularity in Iran and there is a need for increasing product to cover the demands of the Iranian consumers and for overseas markets. According to our knowledge, no studies have been done on the Lighvan cheeses to evaluate their microbiological profiles; therefore this project was conducted to study microbiological characteristics of

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Lighvan raw ewe's milk cheese and to identify the main microbial groups of LAB. Moreover, this study could be the basis for the selection of new strains to be used as specific cultures in a large scale production of traditional cheeses.

MATERIALS AND METHODS

Sampling: During autumn of the year 2004 eight samples (1 kg) of cheese were collected from Amjadi cheese producer in Lighvan village after 4 months of ripening and were taken under refrigeration (4°C) to the laboratory for analysis.

Isolation and identification of colonies: Ten grams of cheese was weighed aseptically and were homogenized in 90 mL of a sterile 2% (w/v) Na-Citrate (Merck) solution at 45°C for 1 min to obtain a 1:10 dilution[3]. Successive decimal dilutions were made in sterile 0.1% (w/v) peptone water (Merck). From each dilution 40 μL was plated in triplicates on MRS agar (Merck) according to Miles and Misra[21]. Solid media were prepared by the addition of 1.5% agar and 100 mg L^{-1} of cycloheximide to the media to prevent the growth of yeasts[15]. Mesophilic and thermophilic lactic acid bacteria were isolated after anaerobic incubation (Merck Anaerocult A GasPak anaerobic systems)[19] at 30°C for 3 days and at 45°C for 48 h, respectively[18, 22].

The colonies were randomly picked from plates with 30-300 colonies and several representative isolates displaying the general characteristics of LAB were chosen from each plate for further studies[3,23,24]. For further studies the working cultures were kept on MRS agar slant at 4°C and subcultured every 4 weeks[4,20].

Phenotypic characterization: All isolates were initially subjected to Gram staining and catalase activity test. Colonies and cell morphology characteristics and also gas production from glucose in MRS-fermentation broth containing inverted Durham tube, were afterwards examined and then separation to phenotypic groups was undertaken[19, 25].

Homofermentative, Gram-positive, catalase-negative cocci, capable of growing at 10 and 45°C, were considered as *Enterococcus* and were reconfirmed by additional tests including Sherman test (survival after heating at 60°C for 30 min), the ability to grow at pH 9.6, growth on 40% bile and sensitivity to salt in MRS broth containing 6.5% NaCl[7]. In order to classify this genus to species, carbohydrates fermentation including maltose, raffinose, melezitose and arabinose were tested[4,20].

The homofermentative, Gram-positive, catalase-negative cocci with tetrad cell arrangement were considered as *Pediococcus* and were classified by the fermentation of carbohydrates included maltose, manitol, sorbitol, xylose, ribose, lactose, rhamnose and melezitose[26].

On the facultatively heterofermentative, Gram-positive, catalase-negative rods, the following tests were performed: growth at 15°C, gas production from gluconate, acid production from ribose, manitol, sucrose, lactose, sorbitol, raffinose and melibiose.

The fermentation of each carbohydrates was determined in duplicate in MRS-fermentation broth (Merck) supplemented with 1% of each carbohydrate and containing bromocresol (0.04 g L^{-1}) as a pH indicator[4,20,25] and soft agar used for overlays contained 0.8 g L^{-1} agar[27].

RESULTS AND DISCUSSION

In total, using MRS-agar, 42 predominant colonies were isolated from Lighvan cheese sample incubated at 30 and 45°C and identified to genus level on the basis of cell morphology and biochemical tests. The morphological and biochemical data showed a diversity of genus and species isolated from Lighvan raw ewe's cheese samples.

All 42 isolates obtained from Lighvan cheese were Gram-positive and catalase negative. The analysis of colonies revealed a diverse range of LAB that was subdivided into 3 genera: mesophilic *Lactobacillus* (19 isolates), *Pediococcus* (5 isolates) and *Enterococcus* (18 isolates). Table 1 shows that *Lactobacillus* is the most frequent genus in Lighvan cheese (46%), whereas *Enterococcus* is the second frequent (42%) and the *Pediococcus* comes third (12%).

Nineteen isolates out of total 42 isolates were referred to genus *Lactobacillus*. They form small round or lenticular white colonies, which are Gram-positive, catalase-negative, microaerophilic and mesophilic rods in chains. A combination of three different tests including: growth at 15°C, gas production from gluconate and fermentation of ribose (in addition to CO_2 production from glucose) were used to classify the present *Lactobacillus* into three major groups as obligatory homofermenter, facultative heterofermenter and obligatory heterofermenter, based on methods described by Tamime[26]. The above tests revealed that all *Lactobacillus* were belonged to facultative heterofermentative group. This is in agreement with Psoni *et al.*[15] findings who reported that NSLAB and mainly facultatively

heterofermentative lactobacilli reach high cell densities in maturing cheeses. In addition, Rehman *et al.*[16] showed that the dominant non-starter bacteria in cheddar cheese made from raw milk are facultative heterofermenter species of lactobacilli. To identify colonies at species level, the biochemical characteristics were tested. Data in Table 2 demonstrate that all isolates were able to ferment manitol, sucrose, lactose and melezitose but could not ferment raffinose. Considering the fact that all isolated *Lactobacillus* were identified as facultative heterofermenter bacteria it was concluded that the *Lactobacillus* isolated from cheese samples in this study should be one of *L. casei* or *L. plantarum*. Results in Table 2 also demonstrate that the biochemical properties of the isolated *Lactobacillus* does not exactly match to those mentioned as a key in the Bergey's Manual[28], therefore, species identification was not precisely possible, although it could be concluded that these isolates are closely related to the species *L. casei* or *L. plantarum*. Sole use of biochemical tests could not positively classify them at the species level; therefore further genotypic characterization of isolates is needed to determine the distinct species of described genus. Difficulties in identifying these microorganisms isolated from cheese were previously reported by other groups[20,22,24].

Present results are also in accordance with Lopez *et al.*[14] who reported that in the case of Artisan starter free cheese in Spain, mesophilic lactobacilli constitute the majority of NSLAB found in most ripened cheese varieties and the dominant species were *L. casei* and *L. plantarum* and *L. brevis*. The abundance of lactobacilli in Lighvan cheese suggests that these organisms may play an important role in this type of cheese. According to Badis *et al.*[20] for the lactobacilli, the flavor of product was due to fermentation of acetaldehyde, which is regarded as being the most characteristic flavor compound in dairy products.

Five identified isolates of *Pediococcus* formed smooth, round, grayish colonies on MRS agar medium. Microscopic observations in an over night culture revealed a cocci characteristic formed into tetrad. All isolates were identified as homofermentative, as they did not produce gas from glucose, fermented maltose, lactose and ribose and but did not ferment melezitose, rhamnose and xylose. Isolates number 1 and 4 were manitol and sorbitol positive but isolates number 2, 3 and 5 were negative. According to biochemical data isolates number 1 and 4 were the same and isolates 2, 3 and 5 showed the same biochemical characteristics (Table 3). When the carbohydrates fermentation patterns of these isolates were compared to the identification key in Bergey's Manual[29], significant differences were noted and identification at the species level by biochemical methods was not exactly possible.

Pediococcus are not used in any dairy starters, though they may grow in maturing cheese and ferment residual lactose over a long period[7,30]. Present results are in accordance with Tserovska[6] who isolated *Pediococcus* from Katyk (raw goat's cheese) in Bulgaria.

Heat resistance at 60°C for 30 min., the ability to grow in MRS broth containing 6.5% NaCl and MRS-agar adjusted to pH 9.6, growth in MRS agar

Table 1: Distribution of LAB genera isolated from Lighvan raw ewe's cheese

Genus name	Percentage of LAB isolates
Lactobacillus	46
Enterococcus	42
Pediococcus	12

Mean data using triplicate culture

Table 2: Biochemical characteristics of *Lactobacillus* isolated from Lighvan raw ewe's cheese

Tests	All isolates of *lactobacillus*
CO_2 production from glucose	+
Growth at 15°C	+
Fermentation of ribose	+
CO_2 production from gluconate	+
Fermentation of manitol	+
Fermentation of sucrose	+
Fermentation of lactose	+
Fermentation of sorbitol	+
Fermentation of raffinose	-
Fermentation of melibiose	+

+ represents positive reaction and - represents negative reaction

Table 3: Biochemical characteristics of *Pediococcus* isolated from Lighvan raw ewe's cheese

Tests/Isolates	1	2	3	4	5
CO_2 production from glucose	-	-	-	-	-
Fermentation of mannitol	+	-	-	+	-
Fermentation of maltose	+	+	+	+	+
Fermentation of xylose	-	-	-	-	-
Fermentation of sorbitol	+	-	-	+	-
Fermentation of lactose	+	+	+	+	+
Fermentation of ribose	+	+	+	+	+
Fermentation of melezitose	-	-	-	-	-
Fermentation of rhamnose	-	-	-	-	-

(+ represents positive reaction and - represents negative reaction)

Table 4: Biochemical characteristics of *Enterococcus* isolated from Lighvan raw ewe's cheese

Tests	All isolates of *Enterococcus*
Growth at 10°C	+
Growth at 45°C	+
Growth in 2% NaCl	+
Growth in 4% NaCl	+
Growth in 6.5% NaCl	+
Survival after 60°C for 30min.	+
Growth at pH: 9.6	+
Growth in bile 40%	+
CO_2 production from glucose	-
Fermentation of maltose	-
Fermentation of raffinose	-
Fermentation of melezitose	-
Fermentation of arabinose	+

(+ represents positive reaction and - represents negative reaction)

containing 40% bile, growth at 45 and 10°C, made it possible to include 18 isolates to be belonging to genus *Enterococcs*. Table 4 shows that all isolates of *Enterococcus* were able to grow in 2 and 4% NaCl, none of them produced gas from glucose and these result confirmed that the isolated *Enterococcus* are homofermentative. Carbohydrates fermentation tests showed that all isolates were arabinose positive but maltose, raffinose and melezitose negative. According to Bergey's Manual[31] all isolates of this genus identified as *Enterococcus faecium*. Our findings are in agreement with Garcia *et al.*[32] and Psoni *et al.*[15] who reported *E. faecium* in San Simon cheese (made from raw cow's milk, without specific starter) and in Batzos (a semi-hard, low fat traditional Greek cheese from raw goat's or ewe's milk, which is produced almost but not exactly the same as Lighvan cheese), respectively. *E. faecium* could play an important role in the development of flavor during the ripening of Lighvan cheese, for they have a high degree of proteolysis activity particularly on casein and other milk proteins, as well as high lipolytic ability[32,33]. Enterococci have certain properties which may help them to become an ideal starter culture (high resistance to adverse factors, high acidification, proteolysis and lipolytic capacity, the ability to produce antimicrobial substance such as bacteriocin and production of typical aroma components such as acetaldehyde, acetoin and diacetyl), therefore they can be used as starters in the manufacture of certain varieties of cheese[33,34]. The only species of *Enterococcus* used officially as a starter culture has so far been *E. faecium*[7,26,32]. *E. faecium* is capable of producing a variety of bacteriocins, called enterocins with activity against *Listeria monocytogenes*[1], this has an important impact on the safety of Lighvan cheese as a cheese usually made from unpasteurised raw milk.

These results showed that a diverse range of NSLAB was present in Lighvan cheese and this could in part be responsible for the desirable quality attributes associated with the product. The beneficial role of Enterococci in development of cheese aroma has led to inclusion of enterococcal strains in certain cultures. *E. faecium* is the only species of *Enterococcus* isolated from Lighvan cheese sample.

The present study further demonstrates the problems in attempting to classify the LAB found in cheese sample using the biochemical methods, in this regard genotypic tests should confirm the phenotypic results.

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