

Identification of *Lactococcus lactis* subsp. Isolates Based on Their Cell Wall Protein Profiles and Plasmid Contents

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Abstract: In this study, among the total 50 lactococci isolates, 44% *L. lactis* subsp. *lactis*, 28% *L. lactis* subsp. *lactis* biovar. *diacetylactis* and 28% *L. lactis* subsp. *cremoris* strains were identified by their phenotypic characterization. According to the cell wall protein patterns, 8.6, 12.5, 14.7, 20.3, 23.4, 25.4, and 31.8 kDa proteins were found identical within 82% of isolates. Moreover 93% of *L. lactis* subsp. *cremoris* strains contained a unique 157.8 kDa protein; however, *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* biovar. *diacetylactis* had no unique protein in their groups. Also a key plasmid profile, which would be used to determine the isolates at subspecies level, could not be obtained.

Key Words: *L. lactis*, cell wall proteins, plasmid

Hücre Duvarı Protein Profilleri ve Plazmid İçerikleri Esas Alınarak *Lactococcus lactis* subsp. İzolatlarının Tanımlanması

Özet: Bu çalışmada, fenotipik karakterizasyonu yapılan 50 adet laktokok izolatının, % 44'ü *L. lactis* subsp. *lactis*, % 28'i *L. lactis* subsp. *lactis* biovar. *diacetylactis* ve % 28'i *L. lactis* subsp. *cremoris* olduğu belirlendi. Hücre duvarı protein profili neticesinde izolatların % 82'sinde 8,6, 12,5, 14,7, 20,3, 23,4, 25,4 ve 31,8 kDa moleküler büyüklükteki proteinlerin ortak olduğu bulundu. *L. lactis* subsp. *lactis* ve *L. lactis* subsp. *lactis* biovar. *diacetylactis* suşları ayırıcı nitelikte proteine sahip değilken, *L. lactis* subsp. *cremoris* suşlarının % 93'ü 157,8 kDa büyüklükte farklı bir protein içerdi. Suşların alt tür düzeyinde belirlenmesinde kullanılabilecek anahtar plazmid profiline ise rastlanmadı.

Anahtar Sözcükler: *L. lactis*, hücre duvarı proteinleri, plazmid

Introduction

Lactococcal strains are essential to milk fermentation, especially in cheese-making processes, providing optimal conditions for curd formation and for development of texture and flavor. It is important for the dairy industry to identify new strains of *Lactococcus lactis* for cheese production. Dairy lactococcal strains are subdivided into *L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis* biovar. *diacetylactis*, and *L. lactis* subsp. *cremoris* based on a few phenotypic tests; growth at 4% NaCl, pH 9.6, at 40 °C

and the ability to hydrolyze arginine (1). Compared to *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* strains are more sensitive to environmental changes, as they are not able to grow at 40 °C, pH of 9.6 as well as in the presence of 4% NaCl and also they cannot hydrolyze arginine (2,3).

Classification according to the phenotypic criteria is often time-consuming and ambiguous. Moreover, distinguishing between subspecies such as *L. lactis* subsp. *lactis* and subsp. *cremoris*, is difficult and there have been

many misclassifications (2,3,4,5). Development of fast, simple, and reliable molecular methods such as PCR analysis (2,6), RAPD (5,7), and RFLP (2,5) has improved and facilitated the discrimination between *L. lactis* subspecies. It is well known that the production of functional proteins in cells, including cell wall proteins, is under strict control (8). Analysis of cell wall proteins has been defined as a useful tool in the identification of species and subspecies of lactobacilli (8,9). However, there are only few reports on cell protein extracts in several strains of *Lactococcus* (3,4,5).

Common features of lactococcal isolates, which are important during the industrial processes, are encoded on plasmids. These include bacteriophage resistance, lactose assimilation, citrate utilization, proteinase activity, and bacteriocin production (10,11,12,13). The potential use of plasmids for classification of *Lactococcus* has attracted more attention with the detection of more stable plasmids compared to other bacteria. According to several studies, some of the plasmids have been found as identifying elements, although exact results have not been reported yet (14,15,16).

In this paper, we report the phenotypic characterization and cell wall protein and plasmid analyses of 50 *L. lactis* subsp. isolates. The aim was to classify isolates on the subspecies level as *L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis* biovar. *diacetylactis* and *L. lactis* subsp. *cremoris*, and to find out indicative proteins or plasmids between subspecies using their protein and plasmid profiles.

Material and Methods

Strains, media, and cultivation conditions

Fifty isolates, which were previously identified as *L. lactis*, were obtained from the culture collection of Ankara University, Department of Biology. All isolates were stored as frozen stocks at -20 °C in M17G, containing 20% (v/v) glycerol. Working cultures were prepared from frozen stock cultures by 2 consecutive transfers in M17G broth at 30 °C.

Identification of *L. lactis* strains at subspecies level

Identification of *L. lactis* at subspecies level was performed according to the criteria of Huggins (1). Morphology of strains was determined by Gram staining. Reddy agar was used to determine NH₃ production from

arginine (17). Elliker broth was used to test the ability to grow at 6.5% NaCl, pH 9.2, and 40 °C. For citrate fermentation, strains were cultivated at 30 °C for 48 h under anaerobic conditions in citrate fermentation medium. The blue colonies were characterized as positive for citrate fermentation ability (18).

Cell-wall protein extraction and analysis

The cell-wall protein extraction and analyses were performed according to the method proposed by Piard et al. (19). Briefly, to 1.6 ml of exponential-phase culture at a given optical density at 600 nm (OD₆₀₀) was added 400 µl of ice-cold 80% (wt/v) trichloroacetic acid (TCA; 16% final concentration). The mixture was kept on ice for 20 min and then centrifuged at 4 °C for 10 min at 11500 g. The resulting pellet was washed twice with 1 ml of acetone, dried, and resuspended in 160 µl per OD₆₀₀ unit TES containing 16 µl lysozyme, 2 µl RNase A. After 30 min incubation at 37 °C, the cells were lysed with 1% sodium dodecyl sulphate (SDS) and subjected to SDS-polyacrilamide gel electrophoresis (PAGE) (12% acrylamide) analysis. Gel was stained with coomassie brilliant blue R250. After the molecular sizes of the cell-wall protein bands of each strains were calculated using the software program of the gel imaging system (KODAK gel logic 1500), the similarity levels between each strains were analyzed with MINITAB 14.0 (Minitab Inc. State College, PA) statistic program based on the Euclidean distance, single linkage amalgamation.

Plasmid profiles

Strains were grown at 30 °C in M17G broth. Cells were harvested from 10 ml of the 3h culture inoculated 10%. Isolation of plasmid DNA was conducted according to the protocol of Anderson and McKay (20). Plasmid DNA of *L. lactis* strains were separated by electrophoresis in 0.7% agarose gels using Tris-acetate buffer (pH 8.0). Gels were stained with ethidium bromide and visualized under UV light.

Results

Phenotypical characterization

Fifty isolates, which were obtained from Ankara University Biology Department Culture Collection, were checked against the initial phenotypical characterization and all of the isolates were confirmed to belong to *Lactococcus* genus as they were Gram positive cocci,

catalase-negative, able to grow at 30 °C in Elliker broth and in the presence of 6.5% NaCl but not at 45 °C. Isolates were further identified at subspecies level according to inability of *L. lactis* subsp. *cremoris* to hydrolyze arginine and ability to grow at 40 °C unlike *L. lactis* subsp. *lactis*. *L. lactis* subsp. *lactis* biovar. *diacetylactis* strains were distinguished with their ability to utilize citrate. Among 50 lactococci isolates, phenotypical characterization of lactococci revealed 44% as *L. lactis* subsp. *lactis*, 28% as *L. lactis* subsp. *lactis* biovar. *Diaceylactis*, and 28% as *L. lactis* subsp. *cremoris*. The table shows the results of physiological and biochemical properties of *L. lactis* isolates.

The cell wall protein profiles

The analysis of cell wall extracts by Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis showed

that *L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis* biovar. *diacetylactis* and *L. lactis* subsp. *cremoris* strains possessed 19-33 proteins with molecular masses 7.6-167.2 kDa. Among these proteins, 8.6, 12.5, 14.7, 20.3, 23.4, 25.4, and 31.8 kDa were considered identical within 82% of isolates. Moreover, 93% of *L. lactis* subsp. *cremoris* strains contained a unique 157.8 kDa protein. However, *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* biovar. *diacetylactis* had no unique protein in their groups (Figure 1). According to the dendrogram analysis, more than 99% similarity was obtained among *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* biovar. *diacetylactis* strains where 78% of the *L. lactis* subsp. *cremoris* strains differed 98% and assembled at different groups. On the other hand, only 4 strains, MLC11, MLD47, MLL48, and MLD46, of all isolates resembled differently from other groups at the level of 97% (Figure 2). Among the groups,

Table. Phenotypic characterizations of *L. lactis* subsp. strains. MLL: *L. lactis* subsp. *lactis*, MLD: *L. lactis* subsp. *lactis* biovar. *diacetylactis*, MLC: *L. lactis* subsp. *cremoris*. C: Coccus.

[illegible]

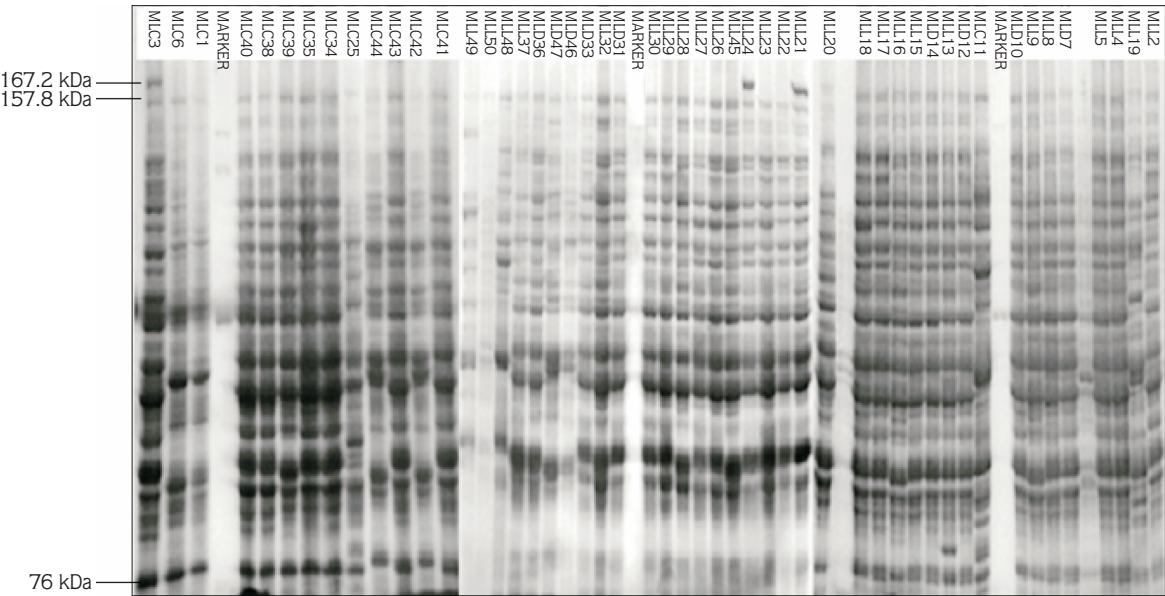


Figure 1. The protein profiles of *L. lactis* subsp. strains. MLL: *L. lactis* subsp. *lactis*, MLD: *L. lactis* subsp. *lactis* biovar. *diacetylactis*, MLC: *L. lactis* subsp. *cremoris*. Marker lanes contains standart proteins (Sigma Chem Co., USA, Cat No: B2787) at molecular sizes of 180.0, 116.0, 58.1, 39.8, 29.0, 20.1, 14.3, and 6.5 kDa.

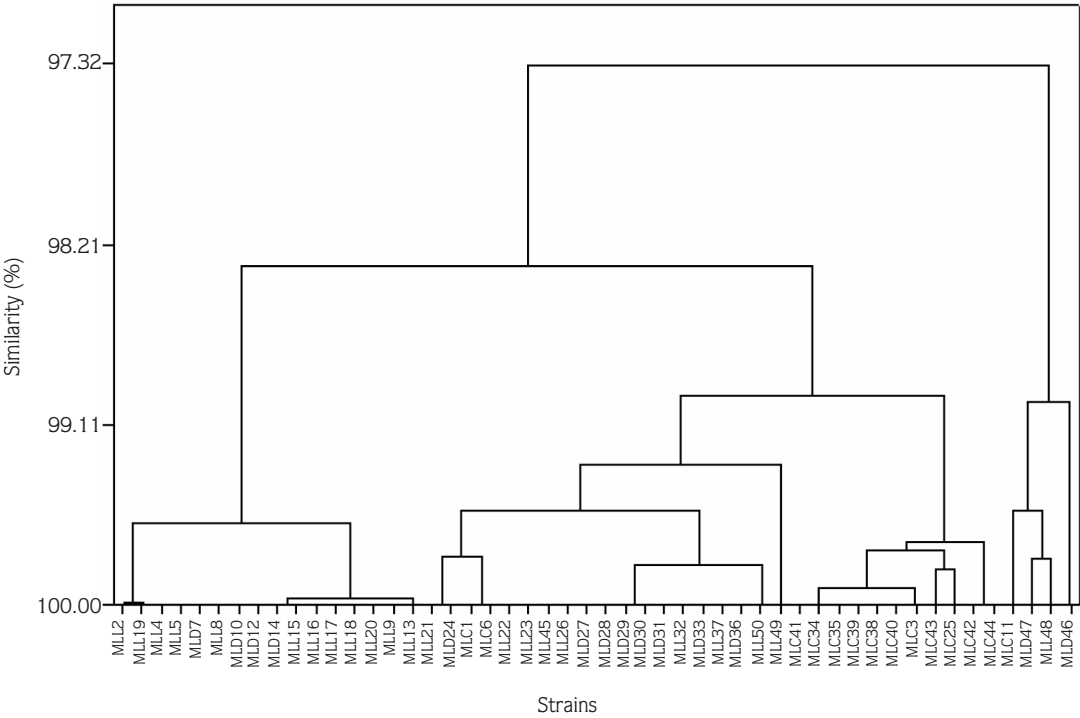


Figure 2. Cell wall protein similarities of *L. lactis* subsp. strains. MLL: *L. lactis* subsp. *lactis*, MLD: *L. lactis* subsp. *lactis* biovar. *diacetylactis*, MLC: *L. lactis* subsp. *cremoris*.

L. lactis subsp. *lactis* biovar. *diacetylactis* MLD7, MLD10, MLD12, and MLD14 were found similar with *L. lactis* subsp. *lactis* MLL4, MLL5, MLL8, MLL9, MLL15, MLL16, MLL17, MLL18, and MLL20 according to their cell wall proteins. Other *L. lactis* subsp. *lactis* strains exhibited the same profiles with the absence of 2 proteins at MLL13, 3 at MLL19, and 4 at MLL2. The second group was formed by high similarities between *L. lactis* subsp. *lactis* MLL22, MLL23, MLL26, MLL32, MLL37, MLL45, and *L. lactis* subsp. *lactis* biovar. *diacetylactis* MLD27, MLD28, MLD29, MLD30, MLD31, MLD33, and MLD36. Only 161.0 kDa extra protein distinguished MLD24 and MLL21 from the second group.

High similarities were also obtained from *L. lactis* subsp. *cremoris* at subspecies level like *L. lactis* subsp. *lactis* strains. Due to the same protein profiles, 3 groups were constituted. The first group contained *L. lactis* subsp. *cremoris* MLC34, MLC38, MLC39, MLC40, and MLC41, the second group MLC42 and MLC44. Members of the 3rd group differentiated from the first group by only 3 proteins. The other *L. lactis* subsp. *cremoris* MLC3, MLC25, and MLC43 were given different protein profiles

not only with groups mentioned above but also between each other. Ninety nine percent high similarity was achieved among the 3 *L. lactis* subsp. *lactis* groups except MLC11 that showed similarity below 98%. It was also found that the profiles of MLC1 and MLC6 were close to the second group (Figure 2).

Plasmid profiles

The numbers of plasmids in *L. lactis* stains were 1 to 10, where the sizes ranged between 2.4 and 41.0 kb, determined using the alkali denaturation method and agarose gel electrophoresis as reported by Anderson and McKay (20). By comparing the plasmid profiles, 2 groups were formed with identical plasmid size and number. The first group, group I, contained 2 isolates (*L. lactis* subsp. *lactis* MLL4 and MLL5) possessing an identical 25.4 kb plasmid. The other group, formed by 3 isolates (*L. lactis* subsp. *cremoris* MLC34, MLC35 and MLC40) contained a unique 28.9 kb plasmid. Sixteen isolates were differentiated including only 1 plasmid. Furthermore a key plasmid profile that would be used to determine the isolates involved in this study could not be obtained at subspecies level (Figure 3).

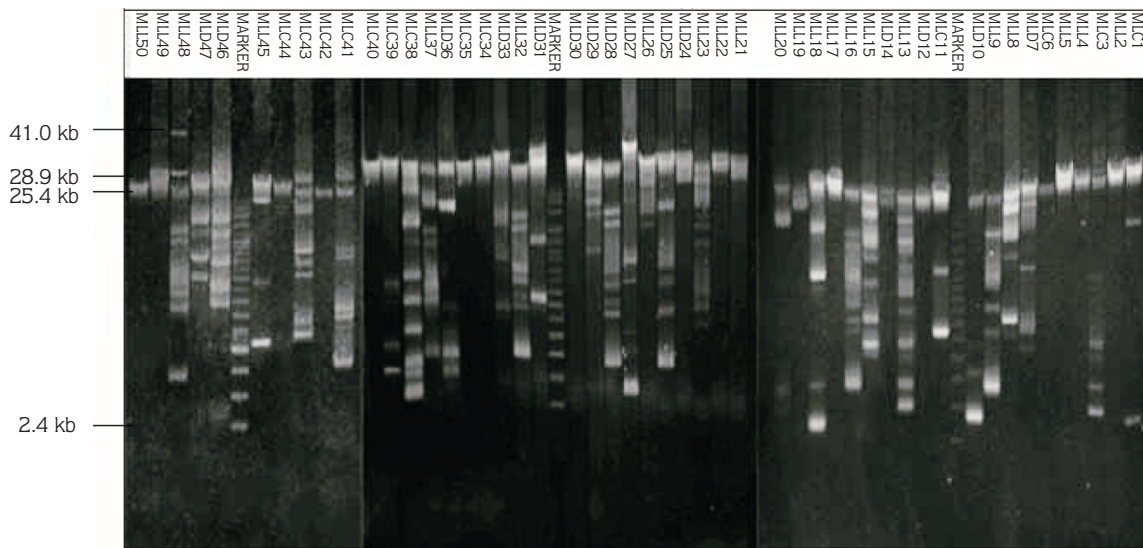


Figure 3. Plasmid profiles of the *L. lactis* subsp. strains. MLL: *L. lactis* subsp. *lactis*, MLD: *L. lactis* subsp. *lactis* biovar. *diacetylactis*, MLC: *L. lactis* subsp. *cremoris*. Marker DNA (Sigma ccc plasmid marker) fragments were 16.2, 14.2, 12.1, 10.1, 8.1, 7.0, 6.0, 5.0, 4.0, 3.0, and 2.1 kb.

Discussion

The classical methods, including morphological, biochemical, cultural, and physiological tests, have been used to identify subspecies of *L. lactis* strains, but these tests are known to possess some disadvantages; for example, they lead to misclassification and are time consuming (2,3). In recent years, use of protein profiles for identification of *Lactococcus* subspecies has been proposed confidently (21,22). However, this method has not become evident to be used for differentiation of *L. lactis* subspecies having industrial importance (3,4,5). In the present study, dendrogram analyses showed that 78% of the *L. lactis* subsp. *cremoris* strains showed 98% similarity in their protein profiles presenting a separate group. However, *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* biovar. *diacetylactis* strains could not be differentiated from each other significantly. Likewise, it has been reported that *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* biovar. *diacetylactis* strains are systematically closely related and differed from each other phenotypically only by the acetoin and diacetyl production ability of *L. lactis* subsp. *lactis* biovar. *diacetylactis* from citrate (1, 2, 3). These pieces of evidence also revealed that analysis by cell wall protein profiles can be performed as a complementary identification method to classical phenotyping and can be successfully used at the differentiation of *L. lactis* subsp. *cremoris* since it has been known as troublesome (2,4,5) in terms of identification. Moreover, the protein at 157.8 kDa molecular size, seen in almost all of the *L. lactis* subsp. *cremoris* cells, might be designated as a major protein for identification. Different proteins have been detected in previously studies, which would have importance at identification of *Lactococcus* subspecies (8,23,24,25). In Figure 2, MLC1, MLC6, MLL46, and MLL47 strains existed differently according to the dendrogram analysis suggesting that these strains might be atypical cells compared to the others. Elliot et al. (4) concluded that classification of lactococcal strains based on biochemical,

cultural and physiological properties were not found attainable especially for differentiating atypical strains.

The presence of plasmids was expected as the diversity of the lactococcal plasmid profiles as reported elsewhere (10,11,12,13). Here we also report that the use of only plasmid profiling is insufficient for differentiation of closely related *L. lactis* subspecies, since only 2 groups having only 2 or 3 members were formed. Therefore plasmid profiles can only be used with other molecular techniques in order to support the results. According to these findings, we are confident to conclude that the members of the 2 groups mentioned above, consisting of MLL4 and MLL5 (group I) and MLC34, MLC35, and MLC40 (group II), regarding their plasmid and protein profiles can be considered as same strains. Furthermore, the variability of the plasmid profiles of the strains can be due to their origin since the samples were collected from different regions of Turkey. Furthermore, plasmids are the most unstable genetic elements influenced by environmental changes because of their possible gain or loss by horizontal or vertical transfer (26,27,28).

In conclusion, cell wall protein results might be promising for differentiating *L. lactis* subspecies, which has the main purpose of increasing the starter culture efficiency. While classical methods used for subspecies identification may require an incubation time of at least 7 days, cell wall proteins can be prepared and SDS-PAGE results can be determined in only a few hours. Analysis of cell wall proteins therefore can be made during the rotation of starter culture processes as routine.

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