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Identification of Genomic Heterogeneity among *Lactococcus lactis* Strains by Plasmid Profiling, PFGE and 16S rDNA Sequence Analysis

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Abstract

Lactococcus lactis strains are used commonly as starters, which contribute to desirable flavour and texture properties known as strain-specific, in dairy industry. Genomic heterogeneity of 30 *L. lactis* strains originating from Turkey and characterized phenotypically were investigated in this study. Plasmid profiling, PFGE and 16S rDNA sequence analyses were performed to determine the genetic variability of strains. High degree of heterogeneity was detected among the *L. lactis* strains. Plasmid profiles of strains showed that compared to the plasmid free control strains, namely; *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *cremoris* MG1614, all tested strains carried one to ten plasmids with molecular size ranging from 1.5 to 41.5 kb. The fingerprints of strains obtained by PFGE from digestion with *Apa*I, *Sma*I and I-*Ceu*I restriction endonucleases of chromosomal DNA's were compared with each other. All strains out of four were grouped into a large cluster A with at least 44% similarity level. The other four strains formed a minor cluster B, distinctively different from major cluster A. PFGE results were confirmed by 16S rDNA sequence analysis and strains included in cluster B were identified as members of different species. These results suggested that morphologic and biochemical methods should be verified by reliable molecular approaches for the purpose of strain typing. Also, PFGE was found suitable to determine genomic differentiations among inter- and intra species.

Key words: *Lactococcus lactis*, genomic heterogeneity, plasmid profiling, PFGE, 16S rDNA sequencing

Introduction

Lactococcus (L.) lactis is a Gram-positive, mesophilic bacterium which can be isolated from various environments such as vegetables, plant surfaces, raw milk and milk products (Nomura *et al.*, 2006; Salama *et al.*, 1995; Ulrich and Müller, 1998). *L. lactis* is regarded as the most famous member of the lactic acid bacteria with low G+C content, and used as a starter culture in commercial milk productions. Due to the fact that it is the main component of fermented milk products, *e.g.*, soft and hard cheeses, butter, sour creams and yoghurt, *L. lactis* strains have a great importance for dairy industry and economy, as well (Fernandez *et al.*, 2011; Parente and Cogan, 2004). While this species is divided into 4 subspecies (subsp. *lactis*, *cremoris*, *hordniae* and *tructae*), only *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* provide flavour and texture compounds for dairy products in industrial processes (Odamaki *et al.*, 2011; Perez *et al.,* 2010). In addition, *L. lactis* subsp. *lactis* has a biovariety called *L. lactis* subsp. *lactis* biovar *diacetylactis*, which can transform citrate into diacetyl as different from the other starter lactococci (Rademaker *et al.*, 2007; Schleifer *et al.*, 1985).

From the past to the present, the subdivision of *L. lactis* species is performed in view of phenotypic properties. *L. lactis* subsp. *lactis* is distinguished from *L. lactis* subsp. *cremoris* with respect to deamination of arginine and growth at 40°C, at pH 9.2, in 4 % NaCl (Mundt, 1986). However, it is accepted that these classification methods aren't sufficient to differentiate between the strains and to accurately find taxonomic positions (Botina *et al.*, 2006; Holzapfel *et al.*, 2001). Morphologic discrimination methods are rarely considered as they have poor reproducibility and discriminatory power (Farber 1996; Rantsiou and Cocolin, 2006; Tran *et al.*, 2011), and moreover they do not express any information about the bacterial genome (Mohania *et al.*, 2008). Therefore, the results obtained from phenotypic tests should be confirmed by molecular typing methods (Charteris *et al.*, 1997; Giraffa and Neviani, 2000; Holzapfel *et al.*, 2001; McCartney, 2002; O'Sullivan, 1999). Unfortunately, it was proved by previous studies that phenotypic and genotypic identification of

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lactococcal strains didn't correspond with each other. This situation causes confusion in the taxonomy of *L. lactis* (Godon *et al.*, 1992; Holzapfel *et al.*, 2001; Kelly *et al.*, 2010; Psoni *et al.*, 2007; Salama *et al.*, 1995).

In the dairy industry, development of new commercial products, which have unique sensorial qualities, is always demanded. It is known that aroma compounds of strains are encoded by extrachromosomal plasmid DNAs (Botina *et al.*, 2006; MacKay, 1983). Besides, many researchers have detected that flavour formation abilities of starters are notably strain-specific (Ayad *et al.*, 1999; Marilley and Casey, 2004; Smit *et al.*, 2000; van Kranenburg *et al.*, 2002; Williams *et al.*, 2001). In order to improve desirable industrial features of substantial starters and develop new industrial strains which will be able to produce different aroma compounds in commercial products, *L. lactis* strains should be well-characterized and evaluated individually by using molecular approaches. Over the last two decades, a lot of strain-specific DNA-based methods for identification and classification of bacteria have been developed such as pulsed-field gel electrophoresis (PFGE), ribotyping, randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP). However, PFGE was found to be more sensitive than the other techniques to differentiate strains based on the principle of separating macrorestriction DNA fragments obtained from restriction enzyme digests (O'Riordan and Fitzgerald, 1997; Yeung *et al.*, 2004). Furthermore, it was also demonstrated by many previous studies that this technique was suitable to distinguish closely related *L. lactis* strains individually (Delgado and Mayo, 2004; Mannu *et al.*, 1999; Mannu *et al.*, 2000; Mannu and Paba, 2002; Tanskanen *et al.*, 1990; Ward *et al.*, 2004). The aim of this study is to determine the genetic heterogeneity of 30 *L. lactis* strains originating from Turkey by using a combination of three molecular typing methods; plasmid profiling, PFGE and 16S rDNA sequencing.

Experimental

Materials and Methods

Bacterial strains, media and culture conditions. 30 of *Lactococcus lactis* strains, which were collected from raw milk and traditional fermented milk products in different regions of Turkey (Akcelik *et al.*, 2000) and characterized by using biochemical and phenotypical tests at subspecies level previously (Ozkalp *et al.*, 2007), were obtained from Prokaryote Genetics Laboratory Culture Collection of Ankara University. These strains were named according to their phenotypic profiles. 10 of them were found at *L. lactis* subsp. *lactis* phenotype, 14 of them were found at *L. lactis* subsp. *lactis* biovar *diacetylactis* phenotype, and finally 6 of them were found at *L. lactis* subsp. *cremoris* phenotype as could be seen in Table I. Except for M10, BLC21 and LL171 strains which are isolated from white cheese, all other 27 strains originated from raw milk. Also, plasmid-free *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *cremoris* MG1614 were used as control strains, and *L. lactis* subsp. *lactis* ATCC 7962 was used as a standard. All strains were grown in M17 broth (Merck, Germany) medium at 30°C for 18 h over the study. Stock cultures were kept in 40% glycerol at –80°C.

Plasmid profiling. Extrachromosomal plasmid DNAs of *L. lactis* strains were extracted as described by Anderson and McKay, (1983), and separated by electrophoresis on 0.7% agarose gels in 1 × TAE buffer at 100 V. Supercoiled DNA ladder (Sigma-Aldrich, USA) was used as a molecular weight marker to determine plasmid sizes. Gels were stained in 1 × TAE containing 0.2 µg/ml ethidium bromide, and visualized under UV light.

Preparation of DNA embedded agarose plugs, restriction digestion and PFGE analysis. PFGE-CHEF-DR III applications guide protocol (Bio-Rad, USA) was modified for isolation intact genomic DNA's of strains before PFGE analysis. An overnight *L. lactis* cultures were diluted in fresh broth until they were standardized between 0.5 and 1 at OD_{600}, and then harvested by centrifugation (10.000 × g, 5 min, 4°C). Pellets were washed twice with CS-Buffer (10 mM Tris pH: 7.0, 20 mM NaCl, 50 mM EDTA pH: 8.0) and resuspended in 100 µl of the same buffer. Equal volumes of 2% low melting grade agarose (BioShop, Canada) was mixed in resuspension. 100 µl mixture was transferred into acrylic disposable plug molds (10 mm × 5 mm × 1.5 mm) and stored till polymerization at 4°C. Cells embedded in agarose were lysed in situ with lysis solution (30 mM Tris pH: 8.0, 5 mM EDTA pH: 8.0, 50 mM NaCl, 10 mg/ml lysozyme) for 4 h at 37°C. Following the incubation agarose plugs were washed with 1 × TE buffer (50 mM EDTA, 20 mM Tris pH: 8.0) so as to eliminate lysis solution. Afterwards, plugs were treated with Proteinase K solution (100 mM EDTA pH: 8.0, 0.2 % sodium deoxycholate, 1% sarkosyl, 1 mg/ml proteinase K) for 18 h at 50°C. Agarose plugs containing intact genomic DNA were washed for 30 min at 50°C; four times with 1 × TE by adding 1 mM NaCl, twice with 1 × TE by adding 1 mM PMSF (phenylmethylsulfonyl fluoride), twice with 1 × TE and finally twice with 0.1 × TE, respectively.

Each DNA embedded agarose plug was cut into four slices. These slices were treated with *Apa*I, *Sma*I and I-*Ceu*I restriction endonucleases, separately. Slices were digested with 25 U of *Apa*I at 37°C and 30 U of *Sma*I at 30°C for 16 h in 100 µl of the 1 × SE-Buffer Y (SibEnzyme, Russia). DNA digestion with 10 U of I-*Ceu*I (New

England Biolabs, UK) was performed also at 37°C for 3 h in 100 µl solution consisting of the enzyme buffer and BSA. After digestions, slices were equilibrated in 0.5 × TBE buffer for 30 min.

Macrorestriction DNA patterns were resolved in 1% pulsed-field certified agarose (BioShop, Canada) in 0.5 × TBE buffer by PFGE-CHEF-DR III System (Bio-Rad, USA). Lambda ladder PFG Marker (New England Biolabs, UK) was used as a molecular size standard. Electrophoresis was performed at 14°C and at 120° angle for three enzymes. For *Sma*I digests, pulse times ranged from 0.5 to 3 s for 5 h, from 5 to 25 s for 6 h and from 40 to 125 s for 8 h at 6 V/cm. Electrophoresis was run at 5 V/cm for both *Apa*I and I-*Ceu*I. For *Apa*I digests, switching times ranged from 0.1 to 5 s for 7 h, from 5 to 35 s for 10 h and from 40 to 125 s for 5 h, on the other hand for I-*Ceu*I digests, switching times ranged from 5 to 125 s for 22 h. After electrophoresis, agarose gels were stained with ethidium bromide (10 µg/ml), visualized under UV light, and documented as TIFF files.

Statistical analysis. Plasmid and PFGE gel images were digitized by *Gel Logic 200 Imaging System* (Kodak Company). Grouping of the plasmid and PFGE patterns were performed by unweighted pair group method using arithmetic average (UPGMA) cluster analysis (Sneath and Sokal, 1973). The similarity matrix was calculated on the basis of Pearson correlation coefficient (r) for plasmid profiles and Dice correlation coefficient for PFGE patterns by NTSYS-pc 2.20 (Rohlf, 1993) computer software.

Genomic DNA isolation, 16S rRNA gene amplifications and sequencing. Genomic DNAs of *L. lactis* strains were extracted by using 'Genomic DNA isolation from Gram-positive bacteria' protocol published by Park, (2007), and stored at –20°C. Oligonucleotide primers described by Beasley and Saris, (2004) were used for 16S rRNA gene amplifications (F: 5'-CCGTCAATTCCTTTGAGTTT-3') (R: 3'-AGAGTTTGATCCTGGCTCAG-5'). PCR was carried out by Thermocycler (Techne, TC-512) in 0.2 µl reaction tubes with 50 µl final mixture volume. Each reaction mixture contained 20 pmol/µl concentration of F and R primers, 2 mM dNTP mix (Fermentas, Finland), 10 µl of 10 × PCR buffer, 25 mM $MgCl_2$, 5U/µl Taq DNA polymerase (Promega, USA) and 3 µl of genomic DNA. Amplification was performed according to the following program: preheating for 5 min at 94°C; 30 cycles of denaturation for 1 min at 94°C; annealing for 15 s at 54°C; extension for 1 min at 72°C and a final terminal extension for 10 min at 72°C. PCR products were examined by 1% agarose gel electrophoresis in 1 × TAE buffer at 100 V. 1 kb DNA ladder (Solis BioDyne, Estonia) was used as a molecular size standard. Amplicons were purified by using Wizard-SV Gel and PCR Clean-Up System (Promega, USA) and sent for sequencing to REFGEN-Biotechnology (METU, Technopolis). Results of the sequencing were aligned by NCBI-BLAST (Basic Local Alignment Search Tool) program to determine the closest known relatives of the 16S rDNA sequence obtained (data not shown).

Results and Discussions

Genotypic diversity of all 30 *L. lactis* strains originated from Turkey was determined by plasmid profiling, PFGE and 16S rDNA sequence analysis. Results of plasmid profiling showed that except for plasmid-free control strains (IL1403 and MG1614), all strains harboured 1 to 10 plasmids with an apparent molecular size ranging from 1.5 to 41.5 kb (Table I). MBLD 59 and MBLD 63 strains contain largest plasmid in size 41.5 kb, whereas MBLD 36 has the smallest one in size 1.5 kb. Fujita *et al.* (1984) reported that *L. lactis* isolates generally carry plasmids whose number vary about 1 to up to 12 with a molecular weight 1.5 from 80 kb. It was detected within a recent study of Fallico *et al.*, (2012) that each lactococcal strain contained from 2 to 10 plasmid patterns ranging in size between 2 and 80 kb. We also obtained identical plasmid profile results in this study for *L. lactis* strains originated from Turkey. Cluster analysis of plasmid profiles demonstrated the genetic variability among isolates (Fig. 1). According to the dendogram, all strains out of MBLD 63 were grouped in two main clusters with about 55% similarity level. It was found that MBLD 63 has unique plasmid profile, so it wasn't located in any cluster. While cluster A branched 7 subgroups for 27 strains, cluster B was composed of only 3 strains, namely; MBLC 47, MBLC 50 and MBLD 54 with a higher similarity level than cluster A. Only MBLC 38 and LL171 have the same plasmid content with one plasmid in size 29.3 kb. Except for these strains, all isolates displayed exactly different plasmid profiles.

Boucher *et al.* (2001) reported that commercial *L. lactis* strains can carry various plasmids which are required to develop important technological features such as lactose fermentation, proteolytic activities and phage resistance. *L. lactis* strains investigated in this study were considered as they can have important properties for dairy industry, owing to numerous plasmid contents of them. On the other hand, it was apparently seen that plasmid profiling wasn't sufficient to identify the strains at subspecies level. Besides, plasmid-free strains, such as IL1403 and MG1614, couldn't be evaluated by this technique. It is also known that plasmids are unstable genetic elements of bacteria, and they are also influenced quickly from environmental factors. They could be got or lost between intra- or inter species via horizontal transfer way (De Vuyst and Degeest,

Table I
Plasmid contents of *L. lactis* strains originating from Turkey with their molecular weights[a]

Strain Code	Number of Plasmids	Molecular weight of Plasmids (kb)
MBLL 1	7	33.1, 29.3, 27.2, 20.3, 12.3, 10.7, 3.2
MBLL 3	9	33.1, 29.3, 27.2, 21.7, 20.3, 15.2, 13.4, 7, 3.2
MBLD 4	8	30.7, 27.2, 23.4, 20.3, 18.6, 15.2, 13.4, 3.2
MBLD 5	8	36.2, 29.3, 27.2, 18.6, 17.4, 12.3, 10.7, 2.5
MBLL 6	8	36.2, 27.2, 20.3, 15.2, 13.4, 10.7, 8, 7
MBLD 7	8	36.2, 30.7, 27.2, 18.6, 15.2, 13.4, 8, 7
MBLL 8	9	33.1, 30.7, 29.3, 27.2, 20.3, 18.6, 12.3, 10.7, 2.5
MBLL 9	9	27.2, 23.4, 20.3, 17.4, 15.2, 12.3, 10.7, 8, 2.5
MBLD 10	5	29.3, 27.2, 20.3, 12.3, 2.5
MBLL 11	6	29.3, 23.4, 17.4, 15.2, 12.3, 8, 3.2
MBLC 15	1	25.7
MBLD 17	9	33.1, 30.7, 27.2, 25.7, 18.6, 17.4, 10.7, 9.2, 2.5
MBLD 19	8	27.2, 25.7, 21.7, 13.4, 12.3, 10.7, 7, 5.5
MBLD 21	9	33.1, 30.7, 25.7, 21.7, 18.6, 10.7, 7, 5.5, 2.5
MBLL 25	4	25.7, 23.4, 15.2, 10.7
MBLL 26	7	30.7, 25.7, 15.2, 13.4, 10.7, 8, 5.5
MBLL 27	8	30.7, 25.7, 18.6, 15.2, 13.4, 10.7, 8, 7
MBLD 35	9	30.7, 27.2, 23.4, 20.3, 12.3, 10.7, 9.2, 5.5, 4.6
MBLD 36	7	30.7, 23.4, 15.2, 8, 3.2, 2.5, 1.5
MBLC 38	1	29.3
MBLC 47	10	36.2, 33.1, 29.3, 21.7, 18.6, 15.2, 12.3, 9.2, 8, 2.5
MBLC 50	8	36.2, 33.1, 30.7, 21.7, 18.6, 12.3, 9.2, 8
MBLD 51	9	36.2, 30.7, 29.3, 20.3, 18.6, 12.3, 10.7, 4.6, 2.5
MBLD 54	10	36.2, 33.1, 29.3, 20.3, 17.4, 12.3, 9.2, 8, 4.6, 2.5
MBLD 55	8	36.2, 30.7, 27.2, 23.4, 18.6, 12.3, 4.6, 2.5
MBLD 59	3	41.5, 27.2, 23.4
MBLD 63	9	41.5, 36.2, 33.1, 27.2, 23.4, 21.7, 5.5, 3.2, 2.5
BLC 21	1	27.2
LL171	1	29.3
M10	5	27.2, 18.6, 17.4, 10.7, 8
ATCC 7962	4	38, 30.7, 27.2, 18.6
IL1403	0	none
MG1614	0	none

[a] The name of strains was assigned according to phenotypical identifications. Expressions: MBLL and LL171: *L. lactis* subsp. *lactis;* MBLC-BLC 21 and M10: *L. lactis* subsp. *cremoris;* MBLD: *L. lactis* subsp. *lactis* biovar *diacetylactis.*

1999; Dieye *et al.*, 2001). In these cases, only typing by plasmid profiling is not so reliable. Thus, we made an elaborate research by using PFGE.

In order to assess the genetic diversity and relatedness among the *L. lactis* strains, 30 lactococcal isolates were exposed to PFGE. Sizes of PFGE-separated macrorestriction fragments obtained from *Sma*I and *Apa*I restriction digestion of control strain IL1403 were determined by Le Bourgeois *et al.* (1992), previously. MG1614 is a plasmid-free derivative of *L. lactis* subsp. *cremoris* MG1363 (Kelly *et al.*, 2000) whose size of restriction fragment sizes were assigned by Le Bourgeois *et al.* (1995). The number of macrorestriction patterns obtained from *Sma*I digest of lactococcal strains was detected between 10 and 24, and each fragment varied from 3 to 950 kb in molecular weight (Fig. 2). Only three strains; namely MBLD 5, MBLD 10 and MBLD 17 had the same *Sma*I band profile. Therefore, these strains were regarded as the same strain. MBLD 21 was differed from them with only presence of a fragment 190 kb in size instead of 180 kb. Consequently, these four strains were considered as having evolved from the same ancestor strain and are closely related. Other lactococcal strains formed unique *Sma*I band profiles. Cluster analysis of macrorestriction patterns generated by *Sma*I digestion of chromosomal DNAs revealed one major and minor group for 33 strains (Fig. 3). Cluster A was a major group including 29 strains with at least 44 % similarity level and subdivided into 6 subgroups. It was apparently seen on the dendogram that strains, which were characterized phenotypically as subsp. *lactis,* were clustered usually together in clusters A1 and A2. In addition, strains characterized as biovar *diacetylactis* were grouped together in clusters A3, A4 and A5. Surprisingly, control strain MG1614 took place in cluster A5. This case made us think that MBLD 54, 55 and 59 get involved in *cremoris* genotype like MG1614. On the other hand, IL1403 and ATCC 7962 were grouped in cluster A2 with other subspecies *lactis* members. Cluster B was a minor group comprising just four strains, namely; MBLC 15, MBLC 18, BLC 21 and M10 at 54% similarity level. In terms of low similarity rates among lactococcal strains, our results were similar with those from a study conducted by Psoni *et al.*, (2007). On the contrary, based on the PFGE patterns of 47 lactococcal total DNA after digestion with *Sma*I restriction enzyme, Kahala *et al.* (2008) defined three major clusters at a similarity level of 75%. Inconsistency between phenotypic and genotypic identification of a strain is a common situation in *L. lactis* taxonomy. In our study, we inferred that strains called MBLD 54, 55 and 59 belonged to the subspecies *cremoris,* although they exhibited similar phenotypic features with biovar *diacetylactis*. This situation was also encountered in a recent study conducted by Demarigny *et al.* (2011). They analysed 184 isolates by REP-PCR and PFGE in order to evaluate diversity of *Lactococcus* population in whey. Consequently, some of the strains were assigned to the *L. lactis* subsp. *cremoris*, despite the fact that they displayed similar phenotypic and REP-PCR profiles with subsp. *lactis.* In another study, Erkus-Kütahya *et al.,* (2011) examined cluster analysis of 82 dairy and nondairy *L. lactis* isolates by AFLP. They observed that strains displaying both subsp. *cremoris* and subsp. *lactis* phenotype were grouped into the same cluster with a similarity level of 80% among strains.

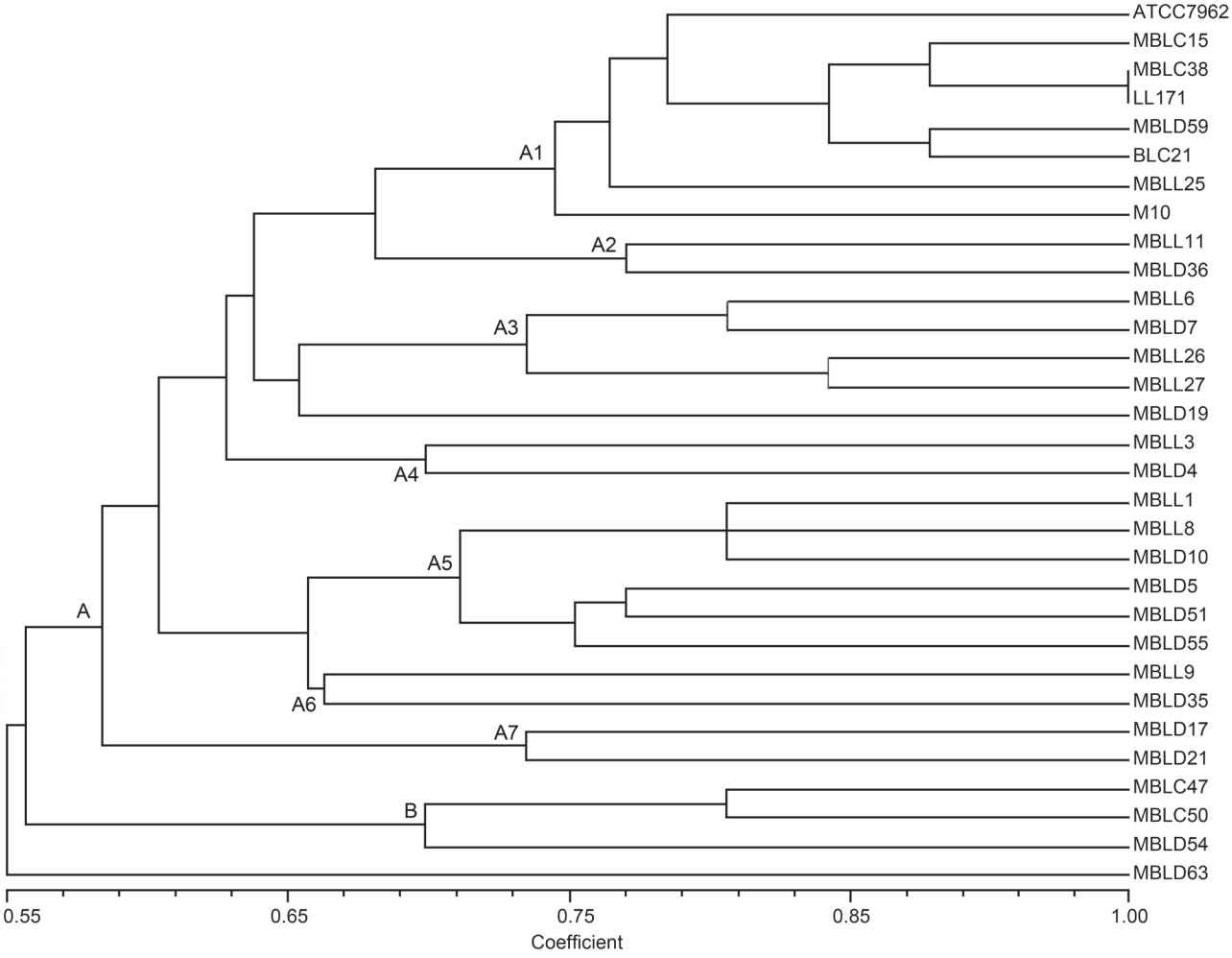

Fig. 1. Clustering of plasmid profiles of *L. lactis* strains by using UPGMA method based on Pearson correlation coefficient.

The macrorestriction fragments of *L. lactis* strains obtained from *Apa*I digestion ranged from 2 to 305 kb in size, and the number of restriction patterns for each strain varied from 15 to 24. The PFGE profile of strains obtained from *Apa*I digestion appeared similar with *Sma*I results. Out of three isolates (BLC 21, MBLC 15 and MBLC 38), all strains were clustered into a large group with a minimal 65% similarity level (data not shown). MBLD 10 and MBLD 17 exhibited the same *Apa*I band profile like *Sma*I digestion. However, it was found that under the electrophoretic conditions performed in this study, the use of restriction endonuclease *Sma*I gave better separated bands than *Apa*I for *L. lactis* strains originating from Turkey. The recognition sites of *Sma*I and *Apa*I endonucleases can be linked up linear plasmid regions integrating into the chromosome within evolutionary processes (Ward *et al.*, 1993). Owing to the contribution of linear plasmids on the *Sma*I and *Apa*I macrorestriction patterns, I-*Ceu*I was used to attain PFGE fragments purely originated from chromosomal DNA. I-*Ceu*I is a homing endonuclease enzyme, digests only 23S rDNA gene regions of the chromosome known as highly protected within the evolutionary processes (Liu *et al.*, 1999). It is also known that *L. lactis* strains have six ribosomal operons with around 2.5 Mb total chromosome length (Kelly *et al.*, 2010; Le Bourgeois *et al.*, 1992). Results obtained from *Sma*I and *Apa*I digestion were confirmed by using I-*Ceu*I restriction endonuclease. The size of I-*Ceu*I fragments of plasmid-free IL1403 was used as a molecular size standard previously described by Le Bourgeois *et al.* (1995). After I-*Ceu*l restriction enzyme digestion of chromosomal DNAs of strains and PFGE treatment, it was observed that 15 of *L. lactis* strains originating from Turkey constituted different I-*Ceu*I fragment numbers varying from 4 to 9 (Fig. 4). Moreover, the total chromosome size of isolates was found to be between 1.98 and 3.3 Mb. I-*Ceu*I fragment sizes of strains were observed in Table II with complete chromosome length.

The results obtained from I-*Ceu*I digestion were suspicious since resulting restriction fragments were not compatible with previous studies as regards *L. lactis* (Kelly *et al.*, 2000; 2010; Le Bourgeois *et al.*, 1995; 2000). 16S rDNA sequencing was applied to support results obtained from I-*Ceu*I digestion. This technique was found as the best by Siezen *et al.* (2011) when it

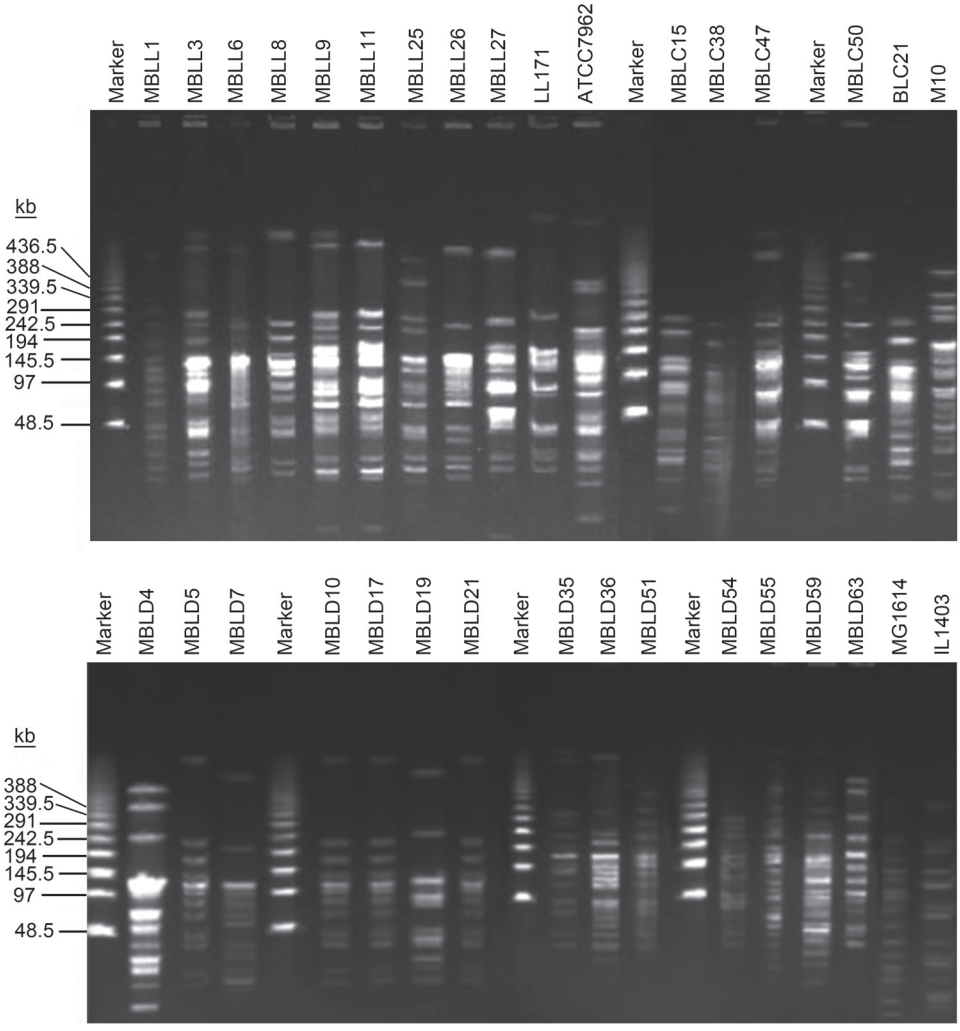

Fig. 2. PFGE patterns of *Sma*I-digested genomic DNA of *L. lactis* strains. Marker: Lambda Ladder PFG Marker (New England BioLabs).

was combined with other methods such as genotypic markers and selected phenotypic tests to describe diversity of *L. lactis* species. It was reported in another study conducted by Pillidge *et al.* (2009) that 16S rDNA gene sequencing gave significant results for *L. lactis* when it was supported by PFGE results. In this study, we also combined 16S rDNA sequence analyses with PFGE. 16S rRNA gene region of strains was amplified by using specific primers. Afterwards, amplicons, which were determined as 940 bp in size for *L. lactis,* were sequenced (data not shown). After comparative 16S rDNA analysis, it was undoubtedly detected that MBLC 15, MBLC 38 and BLC 21 strains, which generated four I-*Ceu*I macrorestriction patterns and were identified previously as *L. lactis* subsp. *cremoris* according to phenotypic tests, are members of *Streptococcus bovis* whose natural habitat is cow udder. It was also determined that strain M10, which formed five I-*Ceu*I fragments and was identified phenotypically as *L. lactis* subsp. *cremoris,* is in a subgroup of *Enterococcus durans*. The other 26 remained strains were identified undoubtedly as *L. lactis.* The increase of ribosomal operon numbers of *L. lactis* strains originated from Turkey can be clarified with large plasmid integrations on recognition site of I-*Ceu*I restriction endonuclease. Integration of large plasmids into chromosome occurs by way of horizontal gene transfers between the strains sharing the same biotope. Also, the increase or decrease of the fragment sizes can relate to gain or loss of moderate prophages, duplicated genes, number of repetitive sequences or mobile elements on chromosome. Such these chromosomal rearrangements could cause chromosomal differentiations of strains belonging to the same genetic lineage (Le Bourgeois *et al.*, 2000). It was also reported by Passerini *et al.* (2010) that PFGE provides a means to monitor genome rearrangements (for instance large inversions or insertions/deletions of mobile genetic elements such as phages and genomic islands) rather than mutations within *L. lactis* species.

In this study, we used plasmid profiling, PFGE and 16S rDNA sequencing in order to identify the heterogeneity and chromosomal diversity of 30 *L. lactis* strains

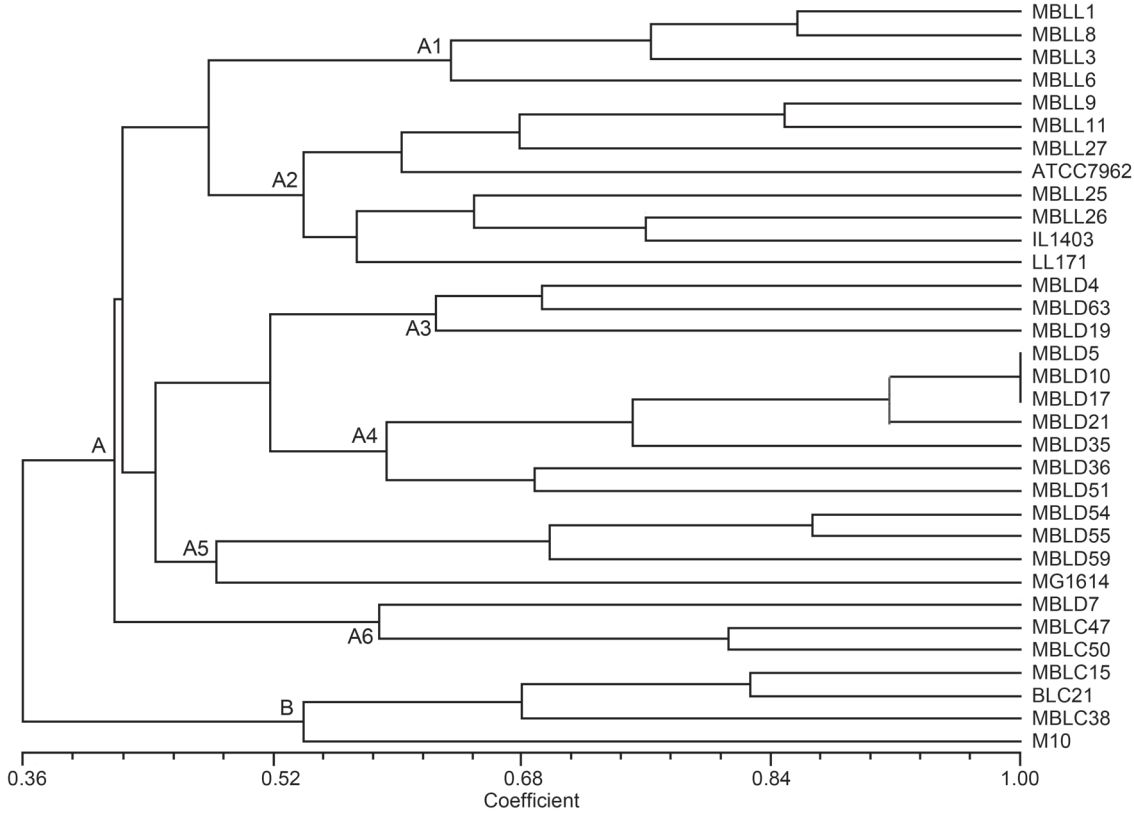

Fig. 3. Clustering of PFGE patterns obtained from *Sma*I digestion of chromosomal DNAs of *L. lactis* strains by using UPGMA method based on Dice correlation coefficient.

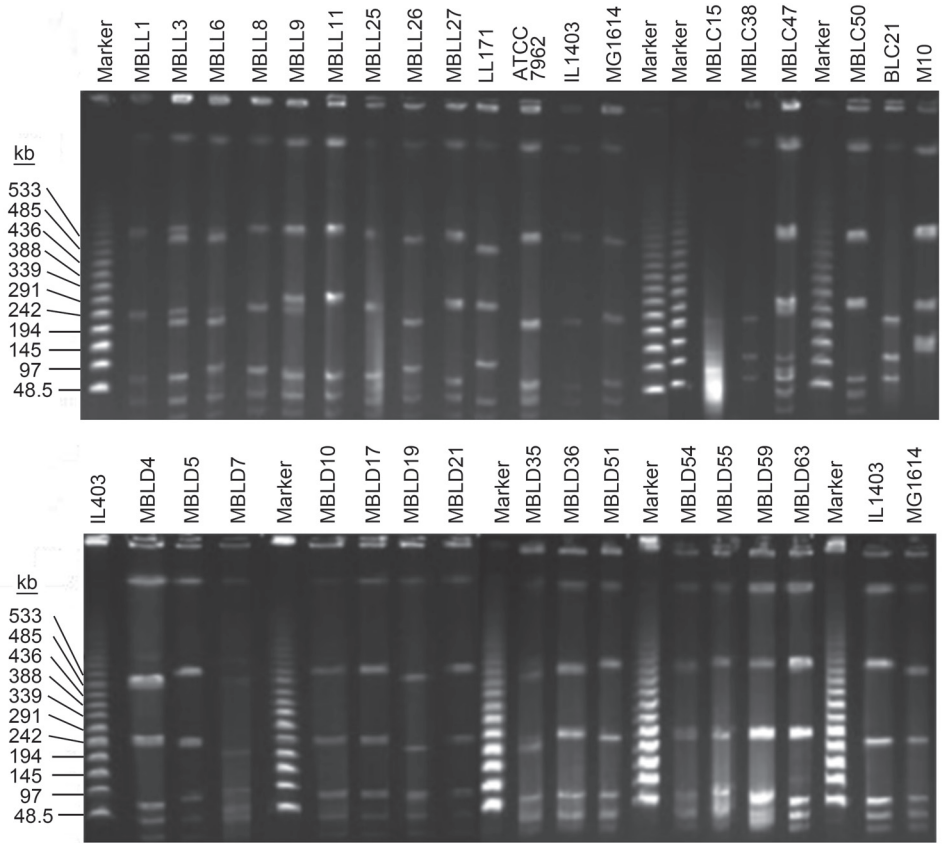

Fig. 4. PFGE patterns of I-*Ceu*I-digested genomic DNA of *L. lactis* strains. Marker: Lambda Ladder PFG Marker (New England BioLabs).

Table II
Size of I-*Ceu*I restriction fragments and total chromosome length of *L. lactis* strains[a]

Strain Code	Sizes of I-*Ceu*I fragments (kb)	Chr. Length (kb)
MBLL1	1550-580-240-80-45-18	2513
MBLL3	1550-580-530-260-230-87-45-18	3300
MBLD4	1480-630-480-240-80-45-18	2973
MBLD5	1480-560-230-87-45-18	2420
MBLL6	1530-550-530-260-230-95-60-45-22	3322
MBLD7	1480-600-480-205-95-60-45	2965
MBLL8	1530-560-280-95-45-22	2532
MBLL9	1520-580-295-260-87-45-18	2805
MBLD10	1480-540-240-87-45-18	2410
MBLL11	1520-600-295-87-45-22	2569
MBLC15	1550-215-135-87	1987
MBLD17	1480-540-240-87-45-18	2410
MBLD19	1480-510-215-87-45-18	2355
MBLD21	1480-550-260-87-45-18	2440
MBLL25	1520-560-280-95-80-45-22	2602
MBLL26	1480-540-240-105-80-45-18	2508
MBLL27	1480-560-280-87-45-18	2470
MBLD35	1520-540-480-230-215-80-45-18	3128
MBLD36	1520-540-240-80-45-22	2447
MBLC38	1550-215-135-87	1987
MBLC47	1530-560-280-240-135-87-80-45-22	2979
MBLC50	1520-560-280-80-45-18	2508
MBLD51	1480-550-230-80-45-22	2407
MBLD54	1550-540-240-230-87-80-45-22	2794
MBLD55	1570-560-240-87-45-18	2520
MBLD59	1550-560-240-87-80-45-22	2584
MBLD63	1500-560-240-80-45-22	2447
BLC21	1530-230-135-80	1957
LL171	1480-510-280-105-45-18	2438
M10	1480-560-280-180-160	2660
ATCC 7962	1520-560-215-80-45-18	2438
IL1403	1480-560-240-80-45-18	2423
MG1614	1530-530-240-80-45-22	2447

[a] The name of strains was assigned according to phenotypical identifications. Expressions: MBLL and LL171: *L. lactis* subsp. *lactis;* MBLC-BLC 21 and M10: *L. lactis* subsp. *cremoris;* MBLD: *L. lactis* subsp. *lactis* biovar *diacetylactis.*

originatingd from Turkey and characterized phenotypically. It was determined that four of them do not belong to *L. lactis* species. So, it was proven with this study that morphological and biochemical tests do not give reliable results for typing. For this reason, results obtained from phenotypic tests should be absolutely verified by molecular typing methods. PFGE was found suitable for both evaluation of genomic heterogeneity among the lactococcal strains and differentiation among interspecies. Also, PFGE results were confirmed by 16S rDNA sequence analysis. High heterogeneity was found among *L. lactis* strains isolated from Turkey. On the other hand, plasmid profiling results did not correspond with PFGE results. We conclude that the plasmid profiling is not sufficient for typing and also for evaluation of genomic heterogeneity of strains. Thus, results obtained from plasmid profiling should be used just to support PFGE results. In conclusion, morphologic and biochemical methods should be combined with molecular approaches for the purpose of strain-specific typing and also development of new starter cultures.

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