

Experimental approaches for identification of indigenous lactococci isolated from traditional dairy products

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Summary

Indigenous lactic acid bacteria contribute to the taste and flavour of traditional dairy products. Therefore, the traditional dairy products might be an interesting reservoir of indigenous lactococcal strains responsible for development of the specific flavour compounds. Consequently, characterized indigenous isolates might be used as a starter culture. The development of molecular techniques provides a new perspective for characterization of the "new lactococcal" strains. However, there is no unique approach suggested for molecular characterization of the indigenous strains associated with the traditional products. The aim of this review is to provide an insight into varieties of experimental approaches applied for molecular characterization of indigenous lactococci associated with traditional dairy products.

Key words: indigenous lactococci, traditional dairy products, identification, molecular characterization

Introduction

The microbiota of traditional cheeses may consist of starter and/or non-starter lactic acid bacteria (LAB), other bacteria, yeasts, and filamentous fungi. Indigenous LAB and secondary microbiota modify the physical and chemical properties of cheese and largely influence its aroma, taste and textural characteristics (Beresford et al., 2001; Jany and Barbier, 2008). LAB isolated from traditional dairy products, or from non-dairy environment, are generally referred to as "wild" or indigenous strains (Wouters et al., 2002). Development of new starter cultures, intended for manufacture of fermented dairy products, usually in-

volves identification and characterization of the indigenous LAB from raw milk cheeses manufactured without the addition of commercial cultures. After isolation, identification and technological characterization, selected indigenous strains may be used as potential starter cultures able to contribute to the sensorial and technological characteristics of industrial or traditional dairy products made from pasteurized milk (Nieto-Arribas et al., 2009). *Lactococcus lactis* is the primary constituent of many industrial and artisanal starter cultures used for the manufacture of a wide range of fermented dairy products, including sour milk, fresh and soft cheeses.

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es and various hard and semi hard cheeses (Vlieg et al., 2006). In several artisanal cheeses produced from raw milk indigenous lactococci were observed as predominant LAB (Bonetta et al., 2008; Florez et al., 2006; Mrkonjić Fuka et al., 2010; Pogačić et al., 2010a), while in the others, lactococci were dominant just in the first period of ripening (Vernile et al., 2008; Dolci et al., 2008). Generally *L. lactis* is recognised as safe organism (Casalta et al., 2008), and it has been exploited also through application as a cell factory for metabolite and protein production (Vlieg et al., 2006; Berlec et al., 2008). It might also be a valuable source of nisin, the most studied bacteriocin used for food preservation (Yoneyama et al., 2008; Babalola 2007; Bravo et al., 2009). There is an increased interest in exploring the potential of new *Lactococcus* strains isolated from different natural ecosystems for the production of the aroma compounds. Except from traditional cheeses and milk, a study by Nomura et al. (2006), demonstrated that indigenous lactococci isolated from plants might also be an alternative to obtain new starters for milk fermentation. Plant-derived *Lactococcus lactis* strains are genetically close to milk derived strains but demonstrated various additional capabilities, as the ability to ferment many additional types of carbohydrates and greater stress-tolerance compared to the milk derived strains (Nomura et al., 2006). A discrepancy between the phenotypic and genetic identification based on amplification of the 16 S rRNA gene has been reported for some *Lactococcus* isolates (Nomura et al., 2006; Vlieg et al., 2006; De la Plaza et al., 2006; Rademarker et al., 2007). Moreover, discrepancy in the molecular identification might also occur because of the preferential amplification of the different target gene or a variable region within the same gene. There are many molecular tools available for genetic characterization of indigenous LAB, but their fundamental details, advantages and disadvantages are not extensively and repetitively described in this review, as that issue has been accomplished and updated in several other studies (Giraffa et al., 2001; Babalola, 2003; Temmerman et al., 2004; Kuchta et al., 2006; Ben Amor et al., 2007; Mohania et al., 2008; Pogačić et al., 2010b).

This review aims to summarize varieties of experimental approaches and designs applied for molecular characterization of indigenous lactococci in

traditional dairy products. Molecular tools are categorized according to their capabilities to identify and differentiate indigenous isolates from genus to strain level.

Molecular characterization of indigenous lactococci

Diversity at the genus to species (subspecies) level

According to recent studies (Cho et al., 2008; Tanigawa et al., 2010), the genus *Lactococcus* is classified into six species: *Lactococcus chungangensis*, *L. garvieae*, *L. lactis*, *L. piscium*, *L. plantarum*, and *L. raffinolactis*. Among these six species, *L. lactis* is especially important because of its use in the manufacture of dairy products. *L. lactis* is further classified as *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *hordniae*, *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* biovar *diacetylactis* (Schleifer et al., 1987; Vlieg et al., 2006). *L. chungangensis* is a new member of the genus *Lactococcus*. It was isolated for the first time from sludge foam and its assignment as a new lactococcal species was confirmed by polyphasic approach combining DNA-DNA hybridization, rep-PCR and biochemical tests (Cho et al., 2008).

Identification of the *Lactococcus* species has been performed by different molecular techniques such as ribosomal ribonucleic acid (rRNA) oligonucleotide probes (Salama et al., 1991), PCR denaturing gradient gel electrophoresis (PCR-DGGE) (Coppola et al., 2001; Bonetta et al., 2008; Mrkonjić Fuka et al., 2010; Pogačić et al., 2010a), multiplex PCR exploiting the diversity of sequences of 16S rRNA gene in *Lactococcus lactis* (Pu et al., 2002), polymorphism of the 16S-23S rDNA spacer region (Blaiotta et al., 2002). Partial ARDRA (Amplified ribosomal DNA restriction analysis) was successfully applied for differentiation of *L. lactis* isolates at subspecies level (Delgado and Mayo, 2003) and for the first time as a culture-independent molecular approach in the identification of indigenous lactococcal community associated with traditional fresh sheep cheese Karakačanski skakutanac (Pogačić et al., 2010a). Amplification of *acmA* gene (Garde et al., 1999) or *PepN* and *PepO* gene (Bensalah et al., 2009) also provided an unequivocal lactococcal assignment. On the basis of PCR amplification of the *acmA* gene, the presence or absence of an additional amplicon of approximately 700 bp correlated with *Lactococcus*

lactis subspecies. *L. lactis* subsp. *lactis* exhibits both the expected 1,131-bp product and the additional amplicon, whereas *L. lactis* subsp. *cremoris* exhibits a single 1,131-bp fragment (Garde et al., 1999).

Diversity at the strain level

Discrimination of indigenous isolates at the strain level is an important step for starter culture selection, because technological and sensorial potential (lactose and citrate fermentation abilities, proteolytic activity, bacteriophage resistance and exopolysaccharide production) can be strain-dependent characteristic (Özkalpa et al., 2007). MLSA (multilocus sequence analysis), (GTG)₅-PCR fingerprinting (Rademarker et al., 2007), PCR-RFLP (PCR-restriction fragment length polymorphism) (Deveau et al., 2003), and MLVA (multiple locus minisatellite typing) (Que´ne´e et al., 2005) have been employed to assess diversity at the strain level of *Lactococcus lactis* isolates. RAPD-PCR (Randomly amplified polymorphic DNA-PCR) (Samaržija et al., 2002), and PFGE (pulse field gel electrophoresis) (Campo et al., 2002) were used for strain differentiation of *Lactococcus lactis* subsp. *cremoris*. Rep-PCR (repetitive element sequence based PCR) was successfully applied for typing of *Lactococcus garviae* isolated from Spanish raw milk cheese (Fernandez et al., 2010). Several reviews have presented details of molecular tools for identification and typing of LAB (Babalola, 2003; Temmerman et al., 2004; Ben Amor et al., 2007; Mohania et al., 2008; Randazzo et al., 2009; Singh et al., 2009). Although PFGE and RAPD techniques have been the most frequently used typing techniques, they also provide limited knowledge about complete genome and specific sequence differences. Taibi et al. (2010) have introduced a microarray-based comparative genomic hybridization (CGH) to investigate genetic variation among *Lactococcus lactis* subsp. *cremoris* strains. The main advantage of the CGH analysis, in comparison with RAPD and PFGE, is that it allows strain grouping and identification of absent or divergent genes involved in metabolism, amino acid biosynthesis, osmoregulation and proteolysis (Taibi et al., 2010). Many bacterial strains harbour plasmids varying in size and numbers and some flavour capabilities might be plasmid coded. Therefore an alternative for strain differentiation can also be a plasmid profiling (Mannu and Paba, 2002). How-

ever, neither this method is universally applicable for all strains, as one of the main drawbacks of the technique is that plasmids can be lost during horizontal gene transfer (Temmerman et al., 2004). In general, there is no “good” or “bad” method; molecular approach can be just more or less complex. Genomics specificity of any strain and discriminatory power of any method cannot be presumed in advance, and it is impossible to select “appropriate” molecular method for typing of specific isolates. Only the complex, polyphasic approach combining two or more techniques can provide far more reliable and objective results. An example of valuable approach was the one presented by Aquilanti et al. (2007) where genetic analysis combining amplification of 16S rRNA gene, enzymatic digestion of amplified 16S rRNA gene with three restriction enzymes and application of RAPD-PCR for assessment of the strain diversity was accomplished. However, Psoni et al. (2007) just demonstrated that application of PFGE and RAPD-PCR to the same isolates did not result in identical grouping of *Lactococcus* isolates. These discrepancies could be explained by different exploration of DNA polymorphism by PFGE and RAPD-PCR. PFGE is based on restriction enzyme polymorphism and analyzes the whole genome, while RAPD analyzes the sequences and extent of polymorphism within regions amplified by random primers (Psoni et al., 2007). Therefore, the application of RAPD-PCR, or any other method as the only typing method, might not provide objective results.

The selected representatives of the strains grouped on the basis of different profiles can be subjected to amplification and sequencing of 16S rRNA gene to obtain an unequivocal species assignment. However, it should be considered that diversity level in the 16S rRNA gene is often insufficient and genetic relationship of closely related species (subspecies) cannot be accurately defined on the basis of 16S rRNA gene sequences (Marzoratti et al., 2008). As an alternative, the intergenic spacer region between 16S and 23S rRNA genes containing variable number of tRNA genes may also be a suitable target for sequencing (Gurtler and Stanisich 1996; Singh et al., 2009). A group of methods, which till recent period have not been so widely applied, like (GTG)₅-PCR fingerprinting (Rademarker et al., 2007), Sau-PCR (Corich et al., 2005), minisatellite

Table 1. Different experimental approaches applied for identification of indigenous lactococci associated with traditional dairy products

Product/basic technological description/origin	Culture media	Total number of isolates	Samples/ sampling periods	Methods used for identification/ characterization	Identified lactococci	References
Salers cheese/ made of raw milk, no starter addition/ central France	M17 agar M17L agar	381	3 farms, 1 cheese per farm/1, 8, 30, 150 day of ripening	Rep-PCR Genus-specific PCR 16S rDNA sequencing	<i>L. lactis</i> subsp. <i>lactis</i> <i>L. garviae</i>	Callon et al., 2004
Toma Piemontese cheese/semi-cooked cheese, no starter/Northern Italy	M17 agar with cyclomeximide	116	6 cheese samples at 30 - 40 days of ripening	16S rDNA sequencing; PCR 16S - 23S rDNA; Gram staining; microscopic examination; catalase test; production of gas; growth at 6.5 % (w/v) NaCl	<i>L. lactis</i> <i>L. garviae</i> <i>L. lactis</i> subsp. <i>cremoris</i> <i>L. lactis</i> subsp. <i>lactis</i>	Fortina et al., 2003
Leben/traditional fermented milk/ Algeria	M17 agar M17 broth	40	41 samples	PCR (amplification of Pep N, Pep O genes)	<i>L. lactis</i>	Bensalah et al., 2009
Pecorino Sardo/semicooked PDO cheese, with or without natural starter/Sardinia	M17 agar	46	Single batch/ 24 h, 1 month	PFGE Plasmid profiling	<i>L. lactis</i>	Mannu and Paba, 2002
Pecorino cheese/ mixing of evening and morning raw milk	M17 agar MRS broth	112	Raw milk, curd, cheese - 7,21,28,42,90, 120 days of ripening; one batch from 3 farms	PCR-ARDRA 16S rDNA sequencing RAPD-PCR	<i>L. lactis</i> subsp. <i>cremoris</i> <i>L. lactis</i> subsp. <i>lactis</i>	Aquilanti et al., 2007
Penamellera cheese/Northern Spain	M17 agar (with 40 ug/mL nalidixic acid PCA	170	3 batches; sampling: 3, 7, 15, 30 day	physiological characterization, acid production, proteinase activity, bacteriophage sensitivity	<i>L. lactis</i> <i>L. rafinolactis</i>	Estepaer et al., 1999
Jben/Moroccan soft white cheese	MRS agar supplemented with sorbic acid	164	8 locations - 18 samples	SDS-PAGE API 50CH Rep-PCR	<i>L. lactis</i> <i>L. garviae</i> <i>L. rafinolactis</i>	Ouadghiri et al., 2005
Batzos/ Greek PDO cheese	M17 broth	40	3 manufacturer of cheese	RAPD-PCR PFGE	<i>L. garviae</i> <i>L. lactis</i> subsp. <i>cremoris</i> <i>L. lactis</i> subsp. <i>lactis</i>	Psoni et al., 2007
Comlek Peniri/ cow's or ewes's milk, no starter addition, ripening/ Central Anatolia (Turkey)	M17 agar MRS broth	90	17 cheeses	PCR-RFLP of 16S rRNA gene -ITS region	<i>L. lactis</i> subsp. <i>lactis</i>	Bulut et al., 2005

polymorphism (Quene et al., 2005) and microarray based CGC analysis (Taibi et al., 2010) might also serve as an alternative for strain typing of indigenous lactococci isolated from traditional products.

Microbiological-molecular approaches applied for characterization of indigenous lactococci isolated from traditional dairy products

Traditional foods include regional and national products manufactured in accordance with cooking traditions (www.truefood.eu). Traditional cheeses represent a cultural heritage and are the result of accumulated empirical knowledge passed from generation to generation. Every traditional cheese is connected to the territory of its origin and to the prevailing pedoclimatic conditions (Alichandis and Polychraniadou, 2008). Typical characteristics of traditional cheeses depend both on local traditions and the indigenous LAB present in raw milk that are selected due to the cheese-making environment (Vernile et al., 2008). There is no unique experimental microbiological and molecular approach proposed for identification of the indigenous lactococci associated with traditional products. In order to present varieties of experimental approaches applied for characterization of indigenous lactococci, Table 1 includes selected specific traditional dairy products.

Salers - traditional French cheese

Salers is a semi hard cheese, ripened for 3 months or more, manufactured from raw cow's milk during grazing summer period on mountain pastures in a limited area in the centre of the France. According to PDO (Protection Denomination of Origin) regulation the cheese is produced exclusively from raw milk collected in a "gerle" (a wood container), which allows a natural microbial flora enrichment and contributes to a specific microbiota. The milk is neither heated nor cooled after milking and no starter culture is added. Callon et al. (2004) collected the cheese samples from three farms at different times of ripening (1, 8, 30 and 150 day) and subjected them to microbiological characterization on the following media: M17 agar incubated at 42 °C for 48 hours, M17 L agar incubated at 30 °C for 48 h or MRS agar incubated at 30 °C under aerobic condition for 48 h. Representative colonies were picked randomly, purified and stored at -20 °C. Total DNA was extracted

from 5 mL of overnight culture in MRS broth, according to the classical phenol-chloroform protocol (Berthier et al., 1999). The total of 381 isolates were classified on the basis of their repetitive DNA sequence amplification profiles (rep-PCR) using primer sets Rep-1R-Dt/REP2-D (Versalović et al., 1991). The densitometric traces of the bands were analysed with BioNumerics software. Calculation of similarity between band profiles was based on Pearson correlation coefficient. The discriminatory power of the rep-PCR was assessed on 49 type strains. Rep-PCR profiles revealed the presence of *L. lactis* subsp. *lactis*. Genus-specific amplification was performed by lactococcal (*L1* and *L2*) primers (Deasy et al., 2000). Partial rDNA from some representative strains of rep-PCR groups were sequenced to confirm presumptive identification. 16S rDNA was amplified with the universal primers WO2 and W18 (Callon et al., 2004), and a fragment of 400 bp of the 16S rDNA was sequenced. The amplified products were analysed using automated DNA sequencer and sequences were compared with those in GenBank DNA database. The only representatives of lactococci confirmed by sequencing were *L. garviae* and *L. lactis* subsp. *lactis*.

Toma Piemontese - traditional Italian cheese

Toma Piemontese is a traditional Italian PDO cheese produced in Piedmont (northern Italy). The cheese is semi cooked, produced from raw milk which has rested for 12 h at 8-10 °C. Without the addition of any natural or selected starter culture, the milk is warmed to temperature of 37-40 °C for 40-60 min and afterward rennet is added. The curd is cut, pressed and drained for 24 h. Cheese is ripened at 6-10 °C and 85 % relative humidity for 30-40 days. The ripening process which lacks temperature and pH control depends entirely on the natural microbial population present in the milk (Fortina et al., 2003). Fortina et al. (2003) performed microbiological and molecular analysis of six cheese samples that were collected after 30-40 days of ripening. Samples were emulsified in sterile 2% (w/v) trisodium citrate, serially diluted in sterile saline solution and plated in duplicate on the M17 agar at 30 °C and 37 °C for 48 hours for mesophilic and thermophilic cocci, respectively. After incubation, randomly selected colonies were purified by two subsequent subcultures and then submitted to

microscopic observation. Genomic DNA was extracted from 100 μ L of overnight cultures. His1 and His2 primer pair (Corroller et al., 1999) were used to amplify histidine biosynthesis operon in *L. lactis*, while pLG-1 and pLG-2 (Zlotkin et al., 1998) to amplify 16S rRNA gene in *L. garviae*. A 500 bp portion of the 16S rRNA gene of some isolates was sequenced.

The 16S-23S rRNA spacer region (RSA) of the 116 coccal isolates was amplified with primers G1 and L1 (Jensen et al., 1993). Five different RSA clusters were obtained, while only cluster 1 consisted of 38 isolates and its profile was characterized by a unique band migrating at approximately 380 bp concerning the spacer region of *L. lactis*. Isolates were further discriminated between the two major subspecies of *L. lactis*. With the primer pair His1 and His2 two different patterns were obtained: 930 bp band for *L. lactis* subsp. *lactis* and 1100 bp band for *L. lactis* subsp. *cremoris*. Some of the isolates which presented the same RSA profile for *L. garviae* were not confirmed by species specific PCR. Further discrimination by the 16 rRNA sequence analysis demonstrated that the isolate belonged to the species *Streptococcus uberis* (Fortina et al., 2003). Discrepancy among the results obtained by different molecular methods required further unequivocal confirmation of species by sequencing of the 16S rRNA gene. Such an approach might be the right tool to clarify the bias of analysis, as application of just one method does not necessarily provide reliable results.

Leben - traditional Algerian fermented milk

Leben is produced from raw cow's, goat's, ewe's and camel's milk using centuries old practises. It is spontaneously fermented milk, which is popular as a daily diet. By conventional cultivation atypical lactococci were identified, which were able to grow in 6,5 % NaCl at pH 9.5 which is untypical for lactococci (Bensalah et al., 2009). As a further step, DNA based method was applied for identification. In total, 41 Leben samples were collected. One millilitre of each sample was transferred into 9 mL Ringer solution and mixed. Aliquotes were plated on M17 agar and incubated at 30 and 42 °C for 2-3 days. Selected colonies were picked out of the agar, enriched overnight in M17 broth and purified twice by streaking on M17 agar. The isolates were frozen

at -80 °C in M17 broth with 15 % glycerol. Cell lysis was performed by modified protocol (Bensalah et al., 2009) and used as a template in PCR experiments. Universal bacterial primer pair U1 and U2 was used to amplify 16S rDNA. The fragments were sequenced and their sequences were homologous to *Enterococcus spp* (98 % of identity) and *Lactococcus lactis* (100 % of identity). From 41 isolates, 38 belong to *E. faecium* group, while just 3 isolates to *L. lactis*. Therefore, further analysis of gene loci was applied to confirm its assignation to genus *Lactococcus*. Isolates were submitted to amplification of gene loci specific for *L. lactis*. Two primer pairs amplifying specifically *Lc. lactis pepN* and *pepO* gene were used, producing amplicons of 600 bp and 500 bp (Bensalah et al., 2009). The isolates amplified expected size of *pepN* and *pepO* gene, confirming assignation to *L. lactis*. However, just 3 isolates, from 41 used in this study were identified as lactococci, and dominance of enterococci was confirmed on M17 media by 16S rRNA gene sequencing. It indicates that reliable identification of isolates should consider a combination of phenotypic and genotypic approaches.

Pecorino Sardo - traditional Italian cheese

Pecorino Sardo is a semi-cooked Italian PDO cheese, ripened for 2 months or more, manufactured in Sardinia from thermally treated or raw full fat ewe's milk. It is mainly manufactured with the addition of autochthonous starter cultures.

Mannu and Paba (2002) analysed a single batch of 24 h and one month old homemade Pecorino Sardo cheese. Cheese samples (10 g) were homogenized in 90 mL sterile 2% (w/v) sodium citrate solution and decimal dilutions of the homogenates were prepared in sterile peptone solution. Aliquots were plated on M17 agar medium and incubated both at 30 °C for 3 days and at 42 °C for two days in anaerobic conditions. Randomly selected colonies (46) were subcultured and checked for purity on M17 agar. Isolates were microscopically observed, Gram stained, and checked for their catalase reaction. The isolates were stored at -80 °C in M17 broth containing 50 % glycerol and then subcultured on M17 medium.

Strain typing of isolates was performed by plasmid analysis and PFGE. For plasmid analysis, extra chromosomal DNA was extracted by alkaline lysis

(Vescovo et al., 1983), and separated by agarose gel electrophoresis. For PFGE analysis, genomic DNA was obtained from 1.5 mL exponential phase culture. The restriction patterns of the DNA were achieved by *SmaI* restriction endonuclease, and the electrophoresis of digested DNA was performed in agarose gel. From 46 *L. lactis* isolates, 26 different profiles were detected by plasmid analysis. Combination of two molecular techniques, plasmid analysis and PFGE, is a more reliable approach for strain typing, as only plasmid based analysis may not be sufficient because some strains lack plasmids. Therefore, plasmid analysis is more suitable as a screening method, to select isolates for further typing (Manu and Paba, 2002).

Aquilanti et al. (2007) applied a different experimental approach for typing of the indigenous isolates from Pecorino cheese. A single batch of Pecorino cheese was sampled after 7, 21, 28, 42, 90 and 120 day of ripening at three farms. Cheese was homogenized and plated according to standard experimental procedures. Single colonies were isolated from M17 agar. ARDRA, sequencing of 16S-rDNA and RAPD-PCR were applied to assess inter- and intra-species diversity. Universal bacterial primer Y1 and Y2 (Young et al., 1991) were used to amplify 16S rRNA gene. Amplicons were digested with *AluI*, *FokI* and *HaeIII* enzymes. Identification of species was confirmed by comparing restriction patterns with those obtained from the type strains, and confirmed by sequencing of the 16S rRNA gene from selected isolates (Aquilanti et al., 2007). A combined approach allowed identifying *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*.

Penamellera - traditional Spanish cheese

Penamellera is a semi-hard traditional Spanish cheese manufactured in the mountainous area of "Picos de Europa" (Asturias region, Northern Spain) from cow's milk, with some seasonal additions of ewe's and goat's milk. The cheese is manufactured traditionally from both the evening and morning milking. Bulk milk is coagulated at 30 °C for about 30-35 min after the addition of commercial rennet. The curd is manually homogenized, salted and slightly pressed. The cheeses are ripened at about 15 °C for a maximum of one month.

Estepar et al. (1999) studied three batches of cheeses that were sampled at three different times of the year (October, February, and April). Cheeses after 3, 7, 15 and 30 days of ripening were analysed. For microbial analysis 10 g of cheese samples were homogenized with 90 ml of a 2 % (w/v) sterilized sodium citrate at 45 °C for 1 min in a stomacher. Decimal dilutions were prepared in sterile 0.1% peptone water and plated on M17 and plate count agar (PCA) agar in duplicate. M17 agar was supplemented with 40 µg mL⁻¹ nalidixic acid. Incubation was performed at 30 °C for 48 h at M17 agar, and 72 h at PCA agar. Eighty five colonies from PCA and 85 colonies from M17 agar plates of different morphology were chosen randomly, purified and maintained at -80 °C in a crioprotective medium. Isolates were examined for colony and cell appearance, catalase activity and Gram staining. Carbohydrate fermentation profiles were determined in MRSF broth (Adsa-Micro), a modified MRS broth without glucose, beef extract and reduced yeast extract content, containing chlorophenol red as a pH indicator, and supplemented with 1 % of each carbohydrate. The majority of the isolates were classified as *Lactococcus lactis* (127), several as *Lactococcus raffinolactis* (7) and *Enterococcus* spp. (19). Lactococci were shown to be prevailing in the Penamellera technology, they were numerically superior during all ripening stages reaching values of 8-9 log CFU g⁻¹. Nevertheless, the phenotypic characterization and differentiation of enterococci from lactococci (growth at 45 °C, at pH 9.6 and in 6.5 % of NaCl) is not considered to be reliable, since there are some strains which show similar characteristics even if they belong to different genera. It would therefore be recommended to use molecular methods to obtain a more reliable characterization of the isolates.

Jben - Moroccan soft white cheese

Moroccan traditional soft white cheese (Jben) has been known and appreciated by consumers for centuries. It is made from both non-pasteurized and pasteurized milk. The cheese is characterized by a total dry matter of about 35 % and a pH lower than 4.5 (Benkerroum et al., 2000). It is widely manufactured and consumed in Morocco, especially during the "Ramadan" fasting month (Ouadghiri et al., 2005).

Ouadghiri et al. (2005) sampled cheeses from eight different regions of Morocco. Eighteen samples were collected and submitted to microbiological and molecular analysis. Ten fold dilutions were plated on MRS agar supplemented with sorbic acid. Incubation conditions were as follows: microaerophilic at 37 °C and aerobic at 30 °C. A total of 164 strains of LAB were isolated and maintained as frozen stocks at -80 °C in MRS broth supplemented with 15 % of glycerol. All the LAB isolates were identified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins. The digital protein patterns were normalized using the software package GelCompar. The isolates were identified by comparison of their patterns with database available at BCCM/LMG Bacteria Collection. Thirty four isolates of LAB were additionally identified by API 50 CH system following the instructions of the manufacturer.

Furthermore, additional molecular approach was applied to all 164 isolates. The bacterial DNA was extracted according to Versalovic et al. (1994), and used as template for rep-PCR genomic fingerprinting (Gevers et al., 2001). In this study sixteen different LAB species were identified, while the genus *Lactococcus* accounted for 27 % of the isolates, and the most frequently isolated species was *Lactococcus lactis* (42 isolates). *Lactococcus garviae* and *Lactococcus raffinolactis* were isolated only once. Discrepancies were observed between genetic and phenotypic approaches since some isolates were identified by API test as representatives of *Lactococcus* genus while rep-PCR attributed them to the *Enterococcus* one. Moreover, isolates identified by API as lactobacilli were subsequently identified as lactococci by rep-PCR (Ouadghiri et al., 2005). The main reason for the discrepancy between phenotypic and genotypic data might be attributed to the loss or gain of plasmids, which led to metabolic inconsistency, as some carbohydrate fermentation capacities are plasmid encoded (Ahrne et al., 1989; Aquilanti et al., 2006).

Batzos - traditional Greek cheese

Batzos is a low-fat traditional PDO Greek cheese made of raw goat's or ewe's milk. Its origin is Western Macedonia (Northern Greece). In the study of Psoni et al. (2003) based on phenotypical identification, indigenous microbiota of Batzos was

found to be dominated by *Lactococcus lactis* subsp. *lactis*. Therefore a further genetic characterization of the lactococcal community was undertaken (Psoni et al., 2007) to obtain more reliable taxonomic data. A total of 40 isolates (winter - 20 isolates; spring - 5 isolates; summer - 15 isolates) from three manufacturers of Batzos cheese were collected. The isolates were obtained from MRS agar and purified by streaking on MRS agar plates and stored in M17 broth supplemented with glycerol (70:30 v/v). The revitalization of the isolates was performed by two successive transfers in the M17 broth before further characterization (Psoni et al., 2007).

RAPD-PCR and PFGE were used for the analysis of the genotypic variability. RAPD-PCR reactions were performed with primers M13 and D8635 (Andrighetto et al., 2001). PFGE restriction enzyme digestion was performed with 20 U of Sma I enzyme. The results obtained by RAPD-PCR and PFGE did not fully overlap. PFGE analysis grouped 39 of 40 isolates as *Lactococcus lactis* subsp. *lactis*, while RAPD grouped 9 isolates as *Lactococcus lactis* subsp. *cremoris*, and 28 as *Lactococcus lactis* subsp. *lactis*. Several isolates did not cluster with any strain type, while one isolate was grouped with *Lactococcus raffinolactis*. This study is a bright example that experiments, based on one method only, do not always provide reliable results. However, even in this case further approaches should be applied to unequivocally identify isolates which mismatch. Amplification of *acmA* gene (Garde et al., 1999) might be among the best possibilities to unequivocally discriminate *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*.

Comlek peyniri - traditional Turkish cheese

Comlek peyniri is a traditional Turkish cheese produced in the Central Anatolia. The fermentation is exclusively based on the activity of the indigenous LAB, as it is manufactured without the addition of a starter culture. The main steps in the cheese production include: clarification of raw milk, heating, cooling, rennet addition, incubation, pressing (whey expulsion), size reduction, salting, and finally ripening in earthen pots, which also serve as the packaging material (Bulut et al., 2005). During the period of ripening, the pots are buried into volcanic ashes, tuff, locally called "kisir", in an upside-down position, in natural caves specific for the region. The rip-

ening process lasts between 3 and 6 months and the consumption time coincides with the winter season. Bulut et al. (2005) collected 17 cheeses for the isolation of indigenous LAB. Presumptive lactococci were isolated from M17 agar after incubation at 30 °C for 3 days. Randomly chosen colonies were cultured in MRS broth and 10 ml of overnight cultures were used for DNA isolation following the protocol of Cardinal et al. (1997). PCR-RFLP of the 16S rRNA gene-ITS (internally transcribed spacer) region was amplified and digested as described previously (Bulut et al., 2005). PCR products were digested with 2 restriction endonucleases, HaeIII and TaqI (MBI Fermentas), and their profiles were compared with those of selected reference strains. Identification of lactococcal isolates revealed just *Lactococcus lactis* subsp. *lactis*, as the only representative of indigenous lactococci in the Comlek cheese.

Conclusion

Indigenous lactococci associated with traditional dairy products can be identified by variety of molecular methods. The experimental approaches selected and presented in this review demonstrated that there is no unique or recommended approach which would be the most suitable for all applications. Additionally, there is no optimal molecular tool for identification and differentiation of indigenous isolates at the strain level. Only the polyphasic microbiological and molecular approach can provide objective and reliable results. Indigenous lactococci, as well as the other indigenous LAB associated with traditional dairy products, represent a natural heritage that needs to be protected and conserved. Such studies are essential since they contribute to the protection of the microbial biodiversity and traditional cheeses. Furthermore, such research might also provide useful strains for industrial and pharmaceutical applications.

Eksperimentalni pristupi za identifikaciju prirodne populacije laktokoka izolirane iz tradicionalnih mliječnih proizvoda

Sažetak

Autohtona populacija bakterija mliječne kiseline prisutna u tradicionalnim mliječnim proizvodima utječe na njihove specifične organoleptičke osobine. Zato tradicionalni mliječni proizvodi također predstavljaju značajan izvor za izolaciju sojeva laktokoka koji potencijalno mogu biti primijenjeni i kao mljekarstva kultura. Razvoj molekularnih metoda otvara nove mogućnosti u karakterizaciji novih sojeva laktokoka. Međutim, ne postoji jedinstveni mikrobiološki i molekularni pristup za identifikaciju prirodne populacije laktokoka prisutne u tradicionalnim mliječnim proizvodima. Cilj ovog rada bio je prikazati mikrobiološke i molekularne eksperimentalne pristupe korištene za identifikaciju prirodne populacije laktokoka tradicionalnih mliječnih proizvoda.

Ključne riječi: prirodna populacija laktokoka, tradicionalni mliječni proizvodi, identifikacija, molekularna karakterizacija

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