

Full Length Research Paper

Evaluation of denaturing gradient gel electrophoresis (DGGE) used to describe structure of bacterial communities in Istrian cheese

Mirna Mrkonjić Fuka^{1,2*}, Viviane Radl², Bojana Bogovič Matijašić³ and Michael Schloter²

¹Department Microbiology, Faculty of Agriculture, University of Zagreb, Svetošimunska 25, 10 000 Zagreb, Croatia.

²Research Unit for Environmental Genomics, Helmholtz Zentrum München, Ingolstaedter Landstrasse 1, Neuherberg, Germany.

³Chair of Dairy Science, Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3, 1230 Domžale, Slovenia.

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Denaturing gradient gel electrophoresis (DGGE) is a powerful method used to study structure of bacterial communities, without cultivation, based on the diversity of the genes coding for ribosomal RNA. However, the results are strongly dependent on the respective target region of the used primer systems. Therefore, three primer pairs that amplify different variable regions of the 16S *rRNA* gene (V1, V3 and V6 to V8) were tested in order to investigate the bacterial diversity existent in Istrian cheese. We found that primer set extremely influenced DGGE analysis. V3 primers were most efficient when 15 cheese associated isolates were resolved by DGGE. However, for Istrian cheese analysis, the best separation and highest number of bands in DGGE patterns were noticed for V6 to V8 primer pairs.

Key words: Denaturing gradient gel electrophoresis, bacterial communities, Istrian cheese.

INTRODUCTION

The culture dependent methods used for characterisation of microbial populations associated with dairy products are often unsuitable as many of these microbes cannot be cultivated using traditional media and incubation procedures due to the long generation times, unknown micronutrients needed for cultivation or syntrophic interactions with other microbes (Quigley et al., 2011). In the last decades, molecular methods have turned out to be a very promising alternative as microbes are characterized based on the sequences of selected house-keeping genes after DNA extraction directly from complex ecosystem. In this respect, the analysis of 16S *rRNA* genes has been

considered as the gold standard (Amann et al., 1995; Ludwig and Schleifer, 1994). With more than 700000 sequences pre-sent in public data basis, the analysis of ribosomal genes allows a clear separation of bacterial phylotypes based on sequence variability (Pruesse et al., 2007). Even with-out detailed sequence analysis of individual amplicons, it is possible to generate a microbial fingerprint which is typical and unique for the respective microbial community.

Molecular fingerprinting methods have become a powerful tool mainly if large numbers of samples are screened and an individual sequencing approach of all samples is not possible. In this respect, denaturing gradient gel electrophoresis (DGGE) is one of the most commonly applied fingerprinting methods to describe structure of bacterial communities based on the diversity of the 16S *rRNA* genes (Ercolini, 2004). Carefully optimized, DGGE is a powerful tool to study the dynamic of microbial communities in any ecosystem. However, the results are strongly influenced by numerous and different

*Corresponding author. E-mail: mfuka@agr.hr or mirna.fuka@yahoo.com. Tel: +385 (0)1 239 4034. Fax: +385 1 239 3881.

Abbreviations: DGGE, Denaturing gradient gel electrophoresis; PCR, polymerase chain reaction.

Table 1. Primers used for amplification of different variable 16S *rRNA* gene regions.

Primer	Composition	Length of PCR product (bp)	16S <i>rRNA</i> gene region	Origin
338fGC 518r	5' –ACTCCTACGGGAGGCAGCAG-3' 5' –ATTACCGCGGCTGCTGCTGG-3'	180	V3	ben Omar and Ampe (2000)
P1V1GC P2V1	5' –GCGGCGTGCCTAATACATGC-3' 5' –TTCCCCACGCGTTACTCACC-3'	89	V1	Cocolin et al. (2001) Rantsiou et al. (2005)
F968GC R1401	5' –AACGCGAAGAACCTTAC-3' 5' –CGGTGT GTACAAGACCC-3'	433	V6-V8	Nubel et al. (1996)

GC, GC clamps.

steps that are included in the procedure. The most critical ones include the selection of the primer system, the gradient of the DGGE gels and the staining procedure used (Ercolini, 2004). Different authors have studied diversity and structural dynamics of microbial communities in dairy products (Cocolin et al., 2001; El-Baradei et al., 2007; Ercolini, 2004; Flórez and Mayo, 2006; Gianino et al., 2009; Ogier et al., 2004) using this approach. However, diverse primer pairs and diverse conditions have been used for the analysis of cheese associated complex microbial communities which leads to the question: Which primer and DGGE condition is optimal for the diversity analysis of cheese microbiota. Therefore, the aim of this study was to evaluate different primer pairs and different gel gradients commonly applied for DGGE analysis in order to find the best conditions to study bacterial community structure in Istrian cheese samples based on extracted DNA and sequence differences of genes coding for 16S *rRNA*. Three primer sets targeting different variable regions of the 16S *rRNA* gene: V1 (P1V1GC- forward (Cocolin et al., 2001), P2V1- reverse (Rantsiou et al., 2005), V3 (338GC- forward/ 518- reverse (ben Omar and Ampe, 2000) and V6 to V8 (984GC- forward/ 1378- reverse (Nubel et al., 1996) were tested using DNA extracted from 15 bacterial species commonly found in cheese as well as DNA directly isolated from cheese samples.

MATERIALS AND METHODS

DNA extraction

Selected *Lactobacillus* strains (*Lactobacillus brevis* DSM 20054, *L. delbrueckii* subsp. *bulgaricus* LMG 6901, *Lactobacillus gasserii* LMG 9203, *Lactobacillus helveticus* DSM 20075, *Lactobacillus paracasei* DSM 5622, *Lactobacillus lactis* DSM 20481 and *Lactobacillus casei* DSM 20011), *Enterococcus* strains (*Enterococcus faecalis* DSM 20478, *Enterococcus durans* DSM 20633, *Enterococcus faecium* DSM 20477, *Enterococcus maloduratus* LMG 10747, *Enterococcus villorum* DSM 15688, *Enterococcus asini* LMG 18727 and *Enterococcus avium* DSM 20679) and *L. lactis* subsp. *lactis* DSM 20481 strains were grown on specific selective media under optimal growth conditions as recommended by German Collection of Microorganisms and Cell Cultures (DSMZ, Braun-

schweig, Germany) and Belgian Coordinated Collections of Microorganisms (BCCM/LMG, Gent, Belgium). DNA from pure cultures was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as recommended by manufacturer.

Total DNA from Istrian cheese samples obtained from two different farms at different ripening stages was extracted and purified using the Maxwell Tissue DNA Purification Kit (Promega, Wisconsin, USA) as recommended by the manufacturer and described in details by Fuka et al. (2010). The DNA concentration was estimated by Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, USA).

Amplification of 16S *rRNA* genes

Different variable regions (V1; V3 or V6 to V8) of the 16S *rRNA* gene were amplified by polymerase chain reaction (PCR) as described by ben Omar and Ampe (2000), Cocolin et al. (2001) and Nubel et al. (1996), respectively. Primer sequences and references are listed in Table 1. PCR products were visualized on agarose gel after staining with ethidium bromide and purified by Qiaquick PCR purification Kit (Qiagen, Hilden, Germany) as recommended by manufacturer. The concentration of PCR products was estimated by Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, USA).

DGGE analysis

DGGE was performed with the D-Code system (Bio-Rad Laboratories, Munich, Germany) as described by Muyzer et al. (1993). 6% (V6 to V8 region) respectively, 8% (V1 and V3 region) polyacrylamide gels (w/v) (ratio of acrylamide and bisacrylamide 37:1) with a denaturant gradient from 40 to 60% were used for analyzing the amplicons (Cocolin et al., 2007). The gels were run at 60°C. V6 to V8 amplicons were run at 60 V for 16 h, whereas V1 and V3 specific PCR products were separated at 120 V after 4.5 and 6 h, respectively (Cocolin et al., 2007). Each lane was loaded with 350 ng of DNA. DGGE gels were stained by SyberGreen I as described by Cocolin et al. (2001).

RESULTS AND DISCUSSION

In this study, the DNA extracted from 15 cheese associated isolates as well as total DNA obtained from two Istrian cheese samples was analyzed by PCR-DGGE in order to optimize and evaluate this method as a tool to study bacterial community structure in Istrian cheese.

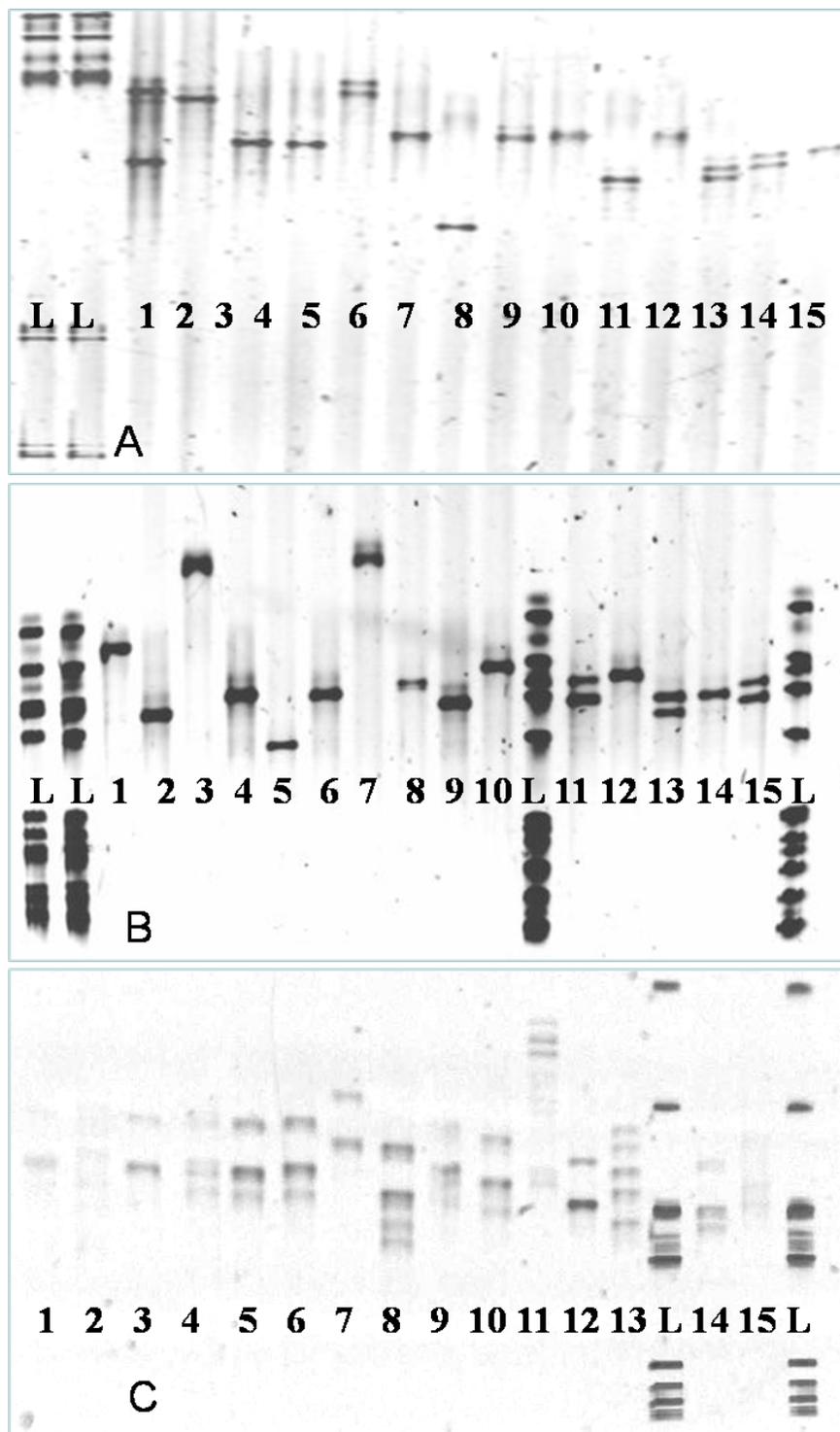


Figure 1. DGGE fingerprinting profiles of variable V6 to V8 (A), V3 (B) and V1 (C) 16S gene regions obtained from extracted DNA from 15 bacterial species. Lanes 1, *L. brevis*; 2, *L. rRNA bulgaricus*; 3, *L. gasseri*; 4, *L. helveticus*; 5, *L. paracasei*; 6, *L. lactis*; 7, *L. casei*; 8, *L. lactis* subsp. *Lactis*; 9, *E. faecium*; 10, *E. faecalis*; 11, *E. villorum*; 12, *E. asini*; 13, *E. maloduratus*; 14, *E. durans*; 15, *E. avium*.

Figure 1 shows DGGE profiles of the PCR products obtained after amplification of the V1, V3 and V6 to V8 re-

gions of bacterial 16S rRNA genes from DNA samples extracted from 15 bacterial strains commonly found in

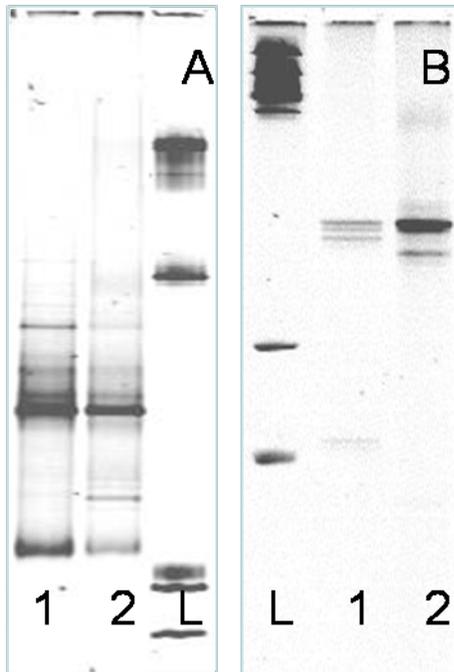


Figure 2. DGGE fingerprinting profiles of amplified V6 to V8 (A) and V3 (B) 16S *rRNA* genes regions obtained from extracted DNA from cheese samples from farms 1 (1) and 2 (2).

dairy products. In the primer pairs that amplified the V3 region, the V6 to V8 region, respectively gave the best results, distinguishing well the 15 strains of different species tested (Figures 1A and B). The high resolution of V3 primer pairs was also confirmed by separating strains belonging to *E. faecalis* and *E. faecium*, the two most abundant enterococcal species found in traditional Mediterranean cheese samples (Čanžek Majhenič, 2006; Poznanski et al., 2004). However, no satisfying results were obtained by applying primers that amplify the V1 region as they generated multiple bands pattern from one species, indicating the presence of multiple operons in some of the strains with sequence heterogeneity in the V1 region (Coenye and Vandamme, 2003). Although, V1 primers were commonly used for diversity analysis of cheese samples (Cocolin et al., 2001, 2007) and gave promising results when enterococcal isolates were applied (Lorbeg et al., 2009), we found them inappropriate for the analysis of complex bacterial communities associated with Istrian cheese due to the occurrence of multiple bands from one strain and they were therefore excluded from further analysis. Investigation of DNA samples from complex microbial communities, extracted directly from Istrian cheese, revealed surprisingly poor separation and low number of distinct bands when primers that amplify the V3 were used (Figure 2B).

In contrast, high resolution and a more complex fingerprinting profiles with higher number of bands was noticed when primers that amplify the V6 to V8 region were used

(Figure 2A). Similar results were obtained in a study of structural dynamics of microbial communities in Provolone del Monaco cheese samples, where higher microbial diversity was obtained by amplifying the V6 to V8 regions as compared to the V3 region (Aponte et al., 2008). A remarkable difference in the DGGE profiles of cheese enterococcal microbiota was also noticed when different primer pairs were used (Lorbeg et al., 2009), demonstrating the need of careful selection of primers when complex microbial communities or even a certain cheese associated microbial population is investigated (Pogačić et al., 2010). However, these results might be highly specific for cheese samples and cannot be generalized for other food products (Cocolin et al., 2007). Our results indicate that the selection of appropriate primer pairs is the crucial step for successful DGGE analysis of complex microbial communities based on extracted DNA and sequences differences of *rRNA* genes from cheese samples.

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