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Evaluation of different primers for PCR-DGGE analysis of cheese-associated enterococci

Petra Mohar Lorbeg*, Andreja Čanžek Majhenič and Irena Rogelj

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

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Enterococci represent an important part of bacterial microbiota in different types of artisanal cheeses, made from either raw or pasteurized milk. Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) of ribosomal DNA is currently one of the most frequently used fingerprinting method to study diversity and dynamics of microbial communities and also a tool for microbial identification. Among several primer pairs for DGGE analysis published so far, six primer pairs amplifying different variable regions of 16S rDNA were selected and applied in our DGGE analysis of 12 species belonging to genus *Enterococcus* and eight other bacterial species often found in cheeses (seven lactobacilli and one *Lactoccocus lactis*). When DGGE procedures were optimized, the same set of primers was used for DGGE analysis of five cheese samples. Our study demonstrates that the use of different primer pairs generate significant differences in DGGE analysis of enterococcal population, consequently, appropriate primers regarding the purpose of analysis can be selected. For differentiation and identification of pure enterococcal isolates, primer pair P1V1/P2V1 showed the most promising results since all 12 enterococcal isolates gave distinctive DGGE fingerprints, but with multiple bands patterns; therefore, these primers do not seem to be appropriate for identification of enterococcal species in mixed cultures. Use of primer pairs HDA1/HDA2 and V3f/V3r amplifying V3 region showed better potential for detection and identification of enterococci in mixed communities, but since some bacterial species showed the same fingerprint, for clear identification combination of DGGE and some other method (e.g. species specific PCR) or combined DGGE analysis using two primer pairs generating distinctive results should be used.

Keywords: 16S rDNA; identification; PCR-DGGE.

Enterococci are naturally present in the gastrointestinal tract of humans and animals, in soil, surface waters, on plants and also in foods, especially food of animal origin, such as cheeses and fermented sausages (Franz et al. 1999).

Enterococci are an essential part of the microflora of different artisanal European cheeses. They occur and grow in different types of cheeses produced from either raw or pasteurised milk (Franz, 2003). Despite their historical bad reputation of faecal contaminants, nowadays enterococci are recognised as a normal part of the microbial population of traditional cheeses. Even more, enterococci play an important role in the ripening of some traditional cheeses and contribute to their specific taste and flavour. Enterococci have been successfully used as a significant component of the starter cultures for the production of different European cheeses (Giraffa, 2003); furthermore, in some countries they are used as probiotics, since many strains are able to survive in the gastrointestinal tract and to produce antimicrobial substances, including bacteriocins (Franz et al. 1999).

The identification of genus *Enterococcus* has always been problematic, since there are no definite phenotypic criteria available for clear discrimination of enterococci from other Gram-positive, catalase-negative, coccus-shaped bacteria (Domig et al. 2003). For reliable and fast identification, especially from sources with a heterogeneous microbial population, molecular-based methods are essential (Klein, 2003). DGGE of PCR-amplified 16S rDNA fragments is currently one of the most used methods for studying complex bacterial communities (Muyzer, 1999; Ercolini, 2004; Temmerman et al. 2004).

Selection of the appropriate primer pair is a crucial step for successful DGGE analysis. A lot of reports describe the use of one primer set for analyses of various food samples,

*For correspondence; e-mail: petra.mohar@bfro.uni-lj.si

Table 1. PCR primers used in this study

Primer†	Sequence (5′–3′)	Amplified 16S rDNA region	Position‡	References	$cMgCl_2$ (mM)
P1V1 P2V1	GCG GCG TGC CTA ATA CAT GC TTC CCC ACG CGT TAC TCA CC	V1	41–130	Cocolin et al. 2004	2·5
HDA1 HDA2	ACT CCT ACG GGA GGC AGC AG GTA TTA CCG CGG CTG CTG GCA C	V2–V3	339–539	Walter et al. 2000	2·5
V3f V3r	CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	V3	341–534	Muyzer et al. 1993	2·5
Com1 Com2-Ph	CAG CAG CCG CGG TAA TAC CCG TCA ATT CCT TTG AGT TT	V4–V5	519–926	Ercolini et al. 2003	1·5
U968 L1401	AAC GCG AAG AAC CTT AC GCG TGT GTA CAA GAC CC	V6–V8	698–1418	Cocolin et al. 2001	3
Ec1055 Ec1392	ATG GCT GTC GTC AGC T ACG GGC GGT GTG TAC	V9	1055–1406	Cocolin et al. 2001	3

† A GC clamp was added to each forward primer according to Walter et al. (2000)
‡ In *E. coli* 16S rDNA gene

but only a few reports are focused on using more and different primer pairs for analysing a particular food sample. The PCR primers used in our study were selected from published research describing DGGE analysis of microbial communities in DNA extracted directly from food samples such as cheeses, sausages and probiotic products. Six particular primer pairs were selected according to criteria as follows: different 16S rDNA regions amplification and suitability for DGGE analysis of bacterial population in fermented food/dairy samples. Selected primers were evaluated on DNA of 12 pure enterococci strains and 8 other LAB, most often found in cheeses (7 lactobacilli and 1 *Lc. lactis*). Primer sets were also DGGE analysed on DNA extracted directly from cheese samples and from enterococcal population harvested from cheese, respectively.

Materials and Methods

Bacterial strains, growth conditions and DNA extraction

Type strains of 12 enterococci were selected: *Enterococcus faecalis* LMG 7937^T, *Ent. faecium* LMG 11423^T, *Ent. hirae* LMG 6399^T, *Ent. durans* LMG 10746^T, *Ent. pseudoavium* LMG 11426^T, *Ent. avium* LMG 10744^T, *Ent. malodoratus* LMG 10747^T, *Ent. casseliflavus* LMG 10745^T, *Ent. mundtii* LMG 10748^T, *Ent. asini* LMG 18727^T, *Ent. pallens* LMG 21842^T and *Ent. villorum* LMG 12287^T; and eight other bacterial species often found in cheeses: *Lactococcus lactis* IM 128, *Lactobacillus helveticus* ATCC 15009^T, *Lb. rhamnosus* ATCC 53103, *Lb. acidophilus* ATCC 4356, *Lb. paracasei* IM 307, *Lb. brevis* LMG 6906, *Lb. plantarum* NCDO 1193 and *Lb. casei* DSM 20011 (LMG: Belgian Co-ordinated Collections of Microorganisms; ATCC: American Type Culture Collection; IM: Chair of Dairy Science, Biotechnical Faculty, Slovenia; NCDO: National Collection of Dairy Organisms; DSM: German Collection of Microorganisms and Cell Cultures). Enterococci and lactococci were cultured in M17 broth at 37 °C or 30 °C, respectively, and lactobacilli in MRS broth at 37 °C. DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Wisconsin, USA).

DNA extraction from bulk cells and cheese samples

DNA was extracted from five artisanal cheese samples, made from raw ewes' milk (cheeses 2 and 3) or cows' milk (cheeses 1 and 5). Cheese 4 was made from thermized cows' milk. Cheese samples (10 g) were homogenized with BagMixer® 400 (Interscience, St Nom, France) in 90 ml of 2% (wt/v) K_2HPO_4 (Merck KGaA, Darmstadt, Germany). For DNA extraction directly from cheese, 10 ml of homogenate was pelleted, resuspended in 1 ml H_2O and extraction was performed according to Cocolin et al. (2001). Prior DNA extraction from bulk cells, 100 µl of homogenate was plated on Citrate Azide Tween Carbonate (CATC) agar (Merck KGaA, Darmstadt, Germany) and incubated for 24 h at 37 °C. Outgrown colonies were collected by rinsing with 1 ml of quarter-strength Ringer's solution and DNA from 1 ml of cell suspension was further extracted using the Wizard® Genomic DNA Purification Kit (Promega, Wisconsin, USA).

PCR primers and protocols

The primers used in this study are listed in Table 1. PCR was performed in final volume of 30 µl containing 1X Colorless GoTaq™ Reaction Buffer, GoTaq™ DNA Polymerase (Promega, Wisconsin, USA), 0·1 mM of each dNTP, 10 µM of primers and $MgCl_2$ at the concentrations listed in Table 1. The amplification programme for primer pairs HDA1-GC/HDA2, V3f-GC/V3r, U968-GC/L1401 and Ec1055-GC/Ec1492 was initiated with denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and elongation

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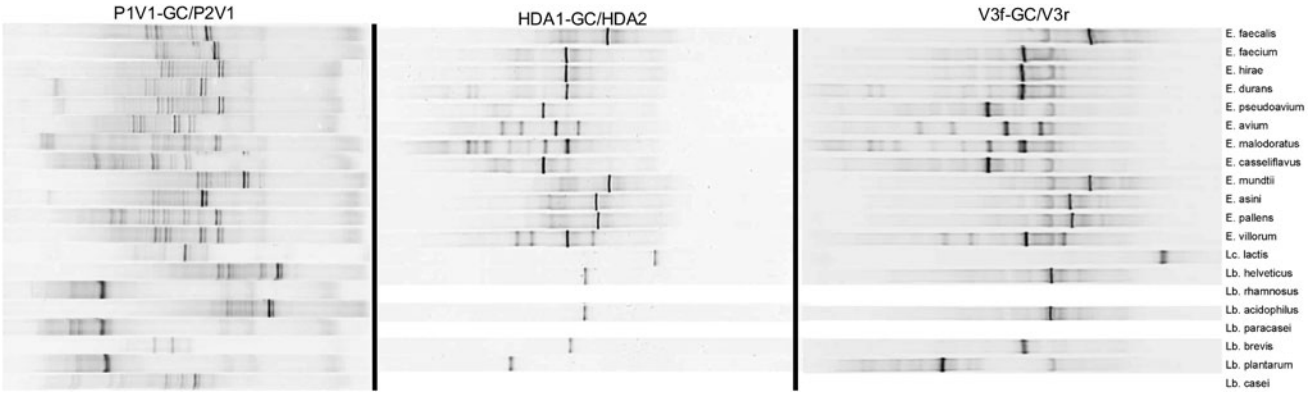

Fig. 1. DGGE profiles of tested strains obtained with primers P1V1-GC/P2V1 (left), HDA1-GC/HDA2 (middle), and V3f-GC/V3r (right).

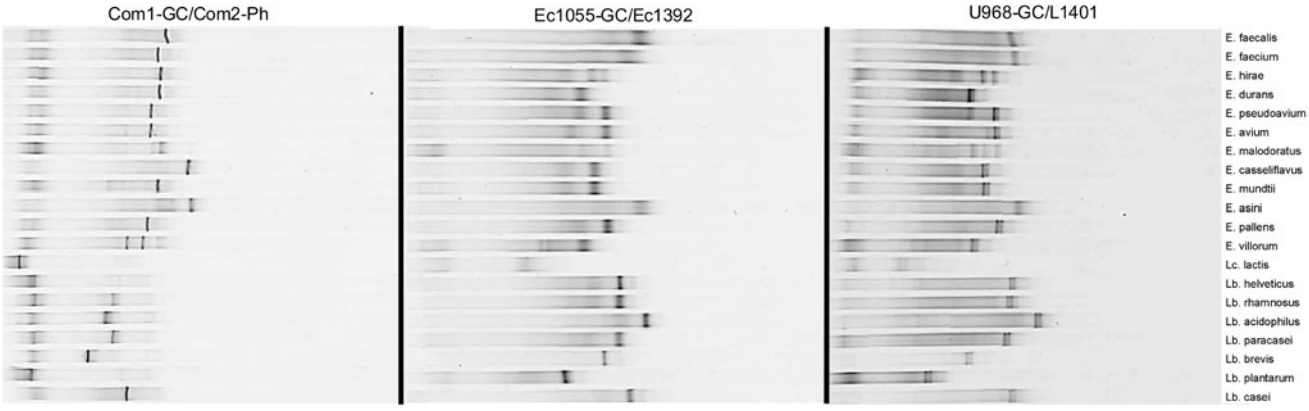

Fig. 2. DGGE profiles of tested strains obtained with primers Com1-GC/Com2-Ph (left), Ec1055-GC/Ec1392 (middle), and U968-GC/L1401 (right).

at 72 °C for 40 s, and terminated with final elongation at 72 °C for 5 min. For primer pair P1V1-GC/P2V1 the annealing was at 54 °C for 30 s and elongation at 72 °C for 30 s; and for primer pair Com1-GC/Com2Ph annealing was at 52 °C for 30 s and elongation at 72 °C for 1 min. PCR products were examined on 1·5% agarose gels, and the reaction mixtures yielding products of expected size were used for DGGE analysis.

DGGE

DGGE was performed with *D GENE* system (Bio-Rad, California, USA) using 16 cm × 16 cm × 1 mm gels. DGGE electrophoresis was performed on 8% (v/v) polyacrylamide gels (acrylamide/bisacrylamide ratio 37 : 1 [Sigma-Aldrich Chemie GmbH, Steinheim, Germany]) with denaturating gradient 30 to 60% for primer pair P1V1-GC/P2V1, 30 to 50% for primer pairs HDA1-GC/HDA2 and V3f-GC/V3r and for the remaining primer pairs 40 to 55% (100% corresponds to 7 M urea [Sigma-Aldrich Chemie, Buchs, Switzerland] and 40% (v/v) formamide [Sigma-Aldrich Chemie GmbH]) in 1 × TAE buffer. Gels were electrophoresed at constant voltage of 130 V for 5–6 h at 60 °C, stained with SYBR safe™ (Invitrogen, California, USA) and photographed with UV illuminator.

Results and Discussion

DGGE analysis of pure strains

Six sets of primers, amplifying different 16S rDNA regions, were applied on DNA of 20 selected strains and 5 cheese samples. They all generated PCR fragments of expected length, which is different from Cocolin et al. (2007) who reported a lack of PCR product for some of the tested strains when primer pair HDA1-GC/HDA2 was used. All PCR fragments were subjected to DGGE analysis and the results are shown in Figs. 1 and 2.

Primer pair P1V1-GC/P2V1, amplifying V1 region of 16S rDNA, generated multiple bands for all of the tested strains, which is in agreement with previous reports (Cocolin et al. 2004, 2007). Since comparison of 16S rDNA sequences of enterococcal population showed the highest heterogeneity within the V1 region, attempts were

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made to find a reason for the appearance of multiple bands. Additional bands with lower intensity could be PCR artefacts that depend upon PCR conditions (Ogier et al. 2002). Therefore, some modifications were made to the PCR programme and the products amplified with different PCR conditions were subjected to DGGE gel. First the temperature influence on the PCR programme was tested by lowering the annealing temperature from 56 to 52 °C in intervals of 2 deg C every three cycles with the additional 25 cycles performed at 50 °C for 1 min. Denaturing step was at 95 °C for 1 min and extension was at 72 °C for 1 min. Initial denaturation and final elongation steps were as described in materials and methods. However, no significant change on DGGE gel was observed. Then, the influence of $MgCl_2$ (1·5, 2·0, 2·5 and 3·0 mM) and DNA (dilution 1:10 and 1:100) concentrations were tested, but again no differences were observed on DGGE gel. Further, the influence of different PCR primers on DGGE was examined, since slightly modified primers and/or primer position can alter the melting behaviour of DNA fragments (Wu et al. 1998). First, modified reverse primer was used for PCR reaction, because original primer differs by two nucleotides from enterococcal 16S rDNA sequences found on NCBI homepage (www.ncbi.nlm.nih.gov). The new primer sequence was 5′-TT**A** CCC ACG **T**GT TAC TCA CC-3′, nevertheless, no change on DGGE gel was observed, which is in agreement with the findings of Boutte et al. (2006). Second, several newly constructed and applied forward and reverse primers, amplifying V1 variable 16S rDNA region, resulted in some changes on DGGE gel, but still multiple bands for single species were present. A possible explanation for these multiple bands phenomena could be the presence of heterogeneous copies of rRNA operons (von Wintzingerode et al. 1997; Muyzer, 1999; Randazzo et al. 2002; Florez & Mayo, 2006) since enterococci possess between five and six rRNA operons (Gurtler, 1999; Zarazaga et al. 2002). For DGGE analysis of the enterococcal population, primers P1V1-GC/P2V1 seem to be more convenient for analysing pure cultures than whole communities since multiple bands for single species could lead to overestimation of the number and variety of bacteria present.

DGGE analysis of fragments amplified with primer pairs HDA1-GC/HDA2, V3f-GC/V3r and Com1-GC/Com2-Ph revealed single band for most of the analysed species. Even if a multiple band pattern appeared, a dominant reference band was always recognisable. In some cases, bands of different bacterial species migrated to the same position on the gel which was expected since there was high sequence similarity; however, it is possible to differentiate between migration distances of bacterial groups. Several authors observed co-migration of bands derived from different species (Walter et al. 2000; Ercolini et al. 2001a; Fasoli et al. 2003; Ogier et al. 2004; Fontana et al. 2005b). Therefore, DGGE with primer pairs HDA1-GC/HDA2, V3f-GC/V3r and Com1-GC/Com2-Ph can be used for initial screening of possible identities of bacterial isolates and then identity could be confirmed with species-specific PCR (Walter et al. 2000). In this way, isolates could be tested only with a couple of primer pairs instead of a complete primer collection. DGGE fingerprints derived with primers V3f-GC/V3r were almost identical to fingerprints derived with primers HDA1-GC/HDA2. These results were somehow expected, since analysis of 16S rDNA showed that both primer pairs amplify the same region.

DGGE separation of amplicons generated with primer pair Ec1055-GC/Ec1392 performed fuzzy bands for all enterococcal strains and for *Lc. lactis* which is because of the presence of multiple melting domains (Wu et al. 1998). This phenomenon was also confirmed with sequence analysis using WinMelt™ software (Bio-Rad Laboratories, California, USA), a program for analysing the melting profile of DNA sequence. Analysis showed that in enterococcal strains primer pair Ec1055-GC/Ec1392 amplifies the fragment with an interior low melting domain consecutively leading to the production of fuzzy bands (Wu et al. 1998). DGGE analysis of tested strains obtained with primers U968-GC/L1401 produced double bands (next to the dominant band one weaker band is present). Our results revealed that the use of this primer pair is not optimal for distinguishing bacterial species belonging to genus *Enterococcus* from other tested bacteria, since the migration distances of different bacterial species are very close together.

DGGE analysis of DNA extracted directly from cheese samples

In order to define which primers are suitable for direct analysis of enterococci in cheese samples, DGGE analysis of 5 cheese samples was performed. DNA was extracted directly from cheese samples as well as from bulk cells harvested from CATC, and PCR amplifications with all six primer pairs were performed. DGGE fingerprints of cheese samples, with addition of marker composed from *Ent. faecalis* and *Ent. faecium*, two enterococcal species most frequently found in cheese, are shown in Figs. 3 and 4.

DGGE analysis of cheese samples with primer pairs HDA1-GC/HDA2 and V3f-GC/V3r (Fig. 3) exhibited similar fingerprints, composed from 4 to 9 clear bands. The presence of band at the same position as *Ent. faecalis* was detected only on fingerprint for cheese 1, while no band was present at the same position as *Ent. faecium*. Using other primer pairs, bands at positions typical for *Ent. faecalis* and *Ent. faecium* were not detected. Several authors reported the lack of bands corresponding to enterococci on DGGE gel from fermented products, although the presence of enterococci was confirmed with other methods (Ercolini et al. 2001b; Fontana et al. 2005a & b; Florez & Mayo, 2006). In contrast, Coppola et al. (2001) and Randazzo et al. (2002 & 2006) were able to show the presence of enterococci with DGGE. Poor detection of enterococci from cheese could be due to their presence in

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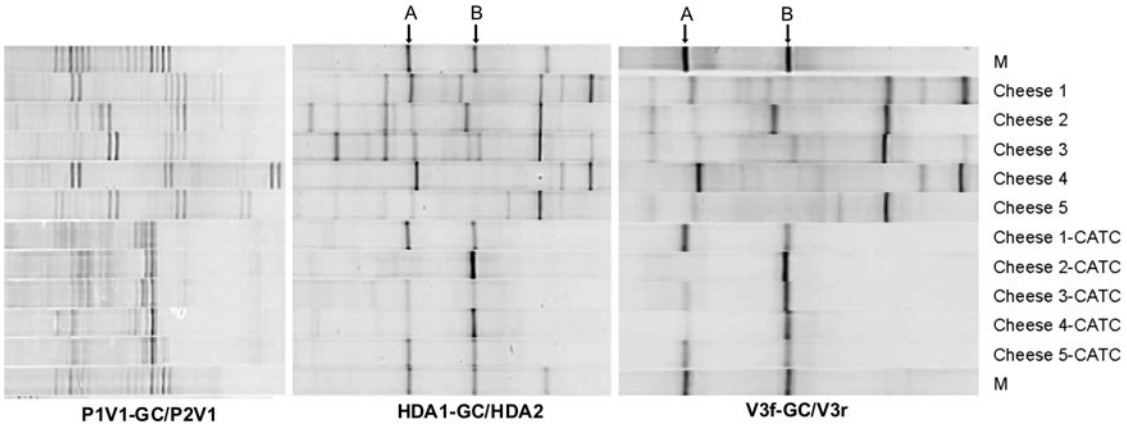

Fig. 3. DGGE profiles of cheese samples and bulk cells obtained with primers P1V1-GC/P2V1 (left), HDA1-GC/HDA2 (middle), and V3f-GC/V3r (right). M-marker composed of *E. faecalis* (A) and *E. faecium* (B) amplicons.

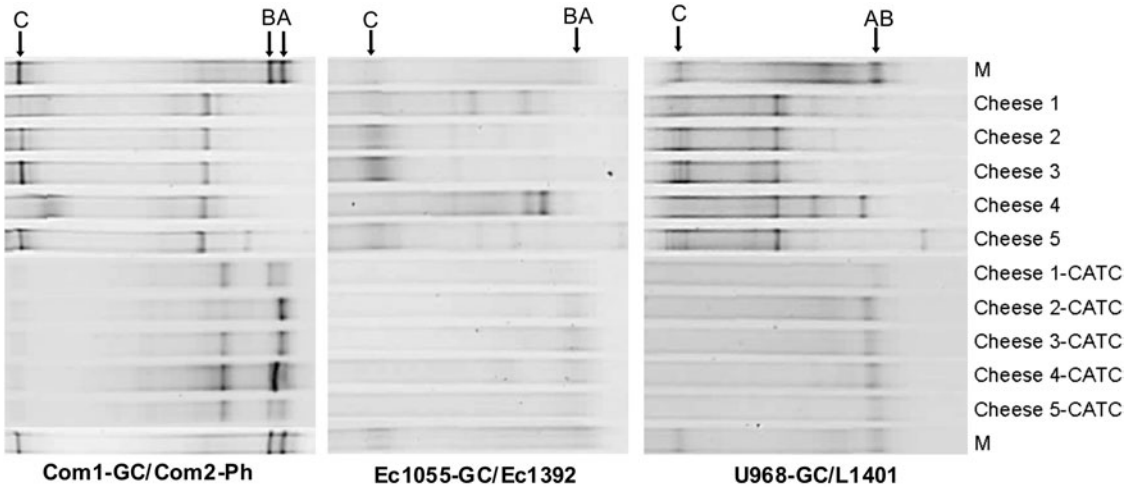

Fig. 4. DGGE profiles of cheese samples and bulk cells obtained with primers Com1-GC/Com2-Ph (left), Ec1055-GC/Ec1392 (middle), and U968-GC/L1401 (right). M-marker composed of *E. faecalis* (A), *E. faecium* (B) and *Lc. lactis* (C).

low numbers, insufficient DNA extraction or preferential PCR amplification. Bacterial species could be detected in mixed populations if they are present in more than 10^4 CFU/g of cheese/food sample (Cocolin et al. 2001; Ercolini et al. 2001b; Temmerman et al. 2003; Fontana et al. 2005b) or when they represent at least 1% of the total microbial population (Muyzer et al. 1993; Fasoli et al. 2003). In the tested cheeses enterococci were present in more than 10^5 CFU/g (determined by plate count on CATC media); with the exception of cheese 4 with only 10^3 CFU/g. Therefore, in most tested cheeses enterococci are present in numbers above detection limit. Another limitation in analysing enterococci from cheese is insufficient DNA extraction. It is very difficult to extract pure DNA from complex samples like cheese, since natural constituents (such as proteins, lipids, salts) can persist until the end of extraction and are found in extract where they might act as inhibitors. Furthermore, not all species have the same sensitivity to lytic agents because of differences in cell wall organisation, particularly Gram-positive species (like enterococci) may not all lyse equally well (Ercolini, 2004; Ogier et al. 2004). Therefore, we preliminarily tested 8 different protocols for DNA extraction from the cheese samples and finally the protocol of Cocolin et al. (2001) was applied, as it yielded more enterococcal DNA than others (data not shown). Another possibility for lack of enterococcal detection in cheese samples is differential/preferential amplification of rDNA genes by PCR. Choice of primers, the varying mol percent G+C composition of 16S rDNA genes, genome size and number of different rRNA operons can affect the differential amplification. DNA-associated molecules which resist standard procedures during DNA purification could be another source of diminished amplification efficiency of

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16S rDNA of Gram-positive bacteria (von Wintzingerode et al. 1997).

DGGE analysis of DNA extracted from bulk cells

Propagation step of cheese samples on CATC media improved detection of enterococcal species. DGGE analysis of microbial population of cheeses grown on CATC media using primer pairs HDA1-GC/HDA2 and V3f-GC/V3r (Fig. 3) exhibited a band at the same position as *Ent. faecalis* for all cheese samples and a band at *Ent. faecium* position for four samples. It has to be pointed out, that *Ent. mundtii* migrated to the same position as *Ent. faecalis*, so the presence of a band at *Ent. faecalis* position could indicate the presence of either or both species. The same is significant for species *Ent. faecium*, *Ent. hirae*, and *Ent. durans*. Similar results were obtained using primer pair Com 1-GC/Com2-Ph (Fig. 4), besides additional band was present for all cheese samples at a position distinct from the positions of the tested enterococcal strains. Primer pair P1V1-GC/P2V1 (Fig. 3) generated several bands for all cheese samples. Since multiple bands arose from a single species, it was not possible to confirm the presence of either *Ent. faecalis* or *Ent. faecium* in tested samples. DGGE analysis revealed that primer pairs U968-GC/L1401 and Ec1055-GC/Ec1392 (Fig. 4) did not differentiate between *Ent. faecalis* and *Ent. faecium*; moreover, detection of either species is vague, since the bands are not sharp.

Conclusions

Our work strongly supports discriminatory potential of PCR-DGGE technique in analysis of enterococcal species. However, regarding the results of our study, it is not simple to find primers for clear differentiation and identification of related bacterial species and also of bacterial species belonging to different genera. Perhaps the selection of genus specific primers or primers for functional or technological genes would enable better discrimination of different enterococcal species. Beside primer selection numerous factors influence on DGGE results for instance protocol of DNA isolation, presence of bacterial population under detection limit, detection of non-viable cells, presence of multiple copies of rRNA operons. Therefore it is worth stressing that DGGE results must be interpreted with great caution.

Mostly DGGE analysis is combined with sequencing of excised bands which is not suitable for identification of related bacterial species since they often harbour the same nucleotide sequence. Besides, sequencing of bands would result in extended time of analysis which is not acceptable for control of food-processing where fast and reliable results are required. For reliable detection of enterococcal population from the food/dairy environment, where viable cells are the ones that count, a combination of both, culture-dependent and culture independent methods, is recommended.

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