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MOLECULAR CHARACTERIZATION OF SEMI-HARD HOMEMADE CHEESE MICROFLORA

JOVČIĆ B, GOLIĆ NATAŠA, KOJIĆ M and TOPISIROVIĆ LJ

Institute of Molecular Genetics and Genetic Engineering, Belgrade

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*This decade has shown an impressive development in the application of molecular techniques based on 16S and 23S rRNA genes to study the microbial diversity in various ecosystems. Microflora of semi-hard homemade cheese was examined in this work. We developed a novel technique for DNA extraction (a bead beating based method) due to high fat content of this cheese. Rapid extraction of DNA from cheese microflora enabled a molecular identification of the LAB (**L**actic **A**cid **B**acteria) strains based on PCR amplification of 16S RNA coding sequences. The specific primers for 16S RNA gene of lactobacilli were used for amplification. The PCR reaction was performed at lower temperature, where the specificity of the annealing reaction was reduced, and lactococcal sequences of 16S RNA genes were also amplified. The results of RFLP analysis revealed that the microflora of Doboj homemade cheese encompases mostly lactococci.*

Key words: cheese, DNA, LAB, PCR, RFLP

INTRODUCTION

A variety of lactic acid bacteria (LAB) are used in the production of a wide range of fermented dairy products where LAB may be added deliberately as a starter culture or are adventitious microbiota selected during the fermentation process. The main purpose of their use is to ensure a proper preservation of the fermented product, but LAB also determine the flavour and texture of the product. Due to industrial importance of these bacteria, studies are being conducted to find a quick and accurate method for enumerating the various populations of cheese microflora. Traditional methods for identifying cheese bacteria which included various culture techniques, bacteriological isolations, biochemical tests, morphological examination, and analysis of volatile and non-volatile fatty acid production were extremely labour-intensive and time consuming. Also, one of the limitations in using these conventional microbiological methods is that only easily cultivable microorganisms are detected. However, those that only grow on specific media, require unknown growth conditions or have obligate interactions with other microorganisms could not be detected using conventional methods. In addition, sometimes those methods can not distinguish the bacteria on the

species level (Bej *et al.,* 1990; Kreader, 1995; Rand *et al.,* 1975; Wren, 1991). In contrast, molecular methods based on the amplification by PCR allow the rapid and specific detection of a wide range of bacterial species and it has become a key procedure for detecting microorganisms. In the case of the analysis of environmental ecosystems, a popular approach has been to amplify 16S rDNA sequences, hopefully containing copies of the gene from all of the species represented in the sample, and to clone, screen and sequence them. Alignment of such sequences with those stored in databanks permits the recognition of which species are represented in the habitat, including those that can not be cultivated by conventional techniques. There are variuos reasons to use rRNA or rDNA genes as markers, including their presence in all bacterial cells, their high degree of sequence conservation which facilitates their detection, the presence of highly variable regions in their sequences which makes them useful to discriminate at (sub)species to higher phylogenetic levels, and the presence of databases containing up to 20000 rRNA sequences from different taxa that facilitate the phylogenetic characterization of cultured and uncultured microbes (Zoentendal *et al.,* 1998). Although sequencing of 16S rRNA/rDNA gene libraries gives valid information about the diversity of the ecosystem of interest, various fingerprinting techniques are also commonly used in research. There are many types of fingerprinting techniques but their essential element is sequence-specific separation of PCR amplicons. Some of those techniques rely on the secondary structures of the single strands (SSCP), while other techniques rely on the melting behaviour of the double stranded amplicon (DGGE, TGGE, TTGE) (Fischer and Lerman, 1979; Hayashi and Yandell, 1993; Rosenbaum and Riesner, 1987). When genetic fingerprinting techniques are used for a microbial community characterization, reliable extraction of DNA and RNA is the most critical step in the whole procedure. An important step of the extraction of nucleic acids is the lysis of microbial cells. Efficient cell lysis may be performed chemically, enzymatically or mechanically depending on the cell envelope of the bacteria of interest. The aim of this work was to establish the method for reliable extraction of DNA from a microbial community of semi-hard homemade cheese from Doboj (Bosnia and Hercegovina). We developed bead beating technique based on mechanical cell disruption, which has been shown to be successful in all kinds of tested samples (Dore *et al.,* 1998; Felske *et al.,* 1996; Harmsen *et al.,* 1995; Ramirez – Saad *et al.,* 1996; Stahl *et al.,* 1998; Wilson and Blitchington, 1996; Zoentendal, 2001).

MATERIALS AND METHODS

Bacterial strains, culture medium and growth conditions

A complete list of bacterial strains used in this study is given in Table 1. The bacteria are aerobic or facultative anaerobic and were cultured, for *E. coli* at 37°C in Luria broth (LB), for *Lactobacillus* sp. and *Lactococcus* sp. at 30°C in MRS and GM17 broth (MERCK, Darmstadt, Germany) respectively.

Table 1. Strains used in this study

Strain	Relevant characteristics	Reference
Lactococcus lactis subsp*. lactis*		
MG1363	plasmid free strain , Lac$^-$, Prt$^-$	de Ruyter *et al*., 1996.
Lactobacillus paracasei subsp. *paracasei*		
BGHN14	cheese isolate	Kojic *et al*., 1991.
Lactobacillus helveticus		
CNRZ32	Laboratory strain PrtH$^+$	Pederson *et al*., 1996.
Escherichia coli		
DH5	F$^-$, *lac, U169(Φ80 lacZ M15), supE44, hsdR17, recA1, gyrA96, endA1, thi-1, relA1*	Hanahan, 1983.

DNA isolation

Isolation of total DNA from cheese

Each cheese sample (0.5 g) was transferred into corex tubes and resuspended in 10 ml of 2% sodium citrate solution containing glass beads. The mixture was incubated at 45°C for 30 min. After incubation the suspension was mixed using a Vortex mixer for 3 min, and then the larger particles were left to settle. The clear supernatant was transferred to clean tubes and centrifuged at 8000 rpm for 10 min at 4°C. The fat layer was removed after centrifugation with sterile glass rods with cotton tips, and then the sample was centrifuged again under equal conditions (10 min, 8000 rpm, 4°C). Most of the supernatant was decanted, and the pellet was resuspended in 1 ml of supernatant and then transferred into the bead beating microtubes containing 180 μl of macaloid and 0.3 g of 0.1 mm zirconium beads (BIOSPEC PRODUCTS, INC., Bartlesville). Then 50 μl of 10% SDS and 150 μl of neutral phenol were added, and the sample was treated for 2 min in a bead beater. After the bead beating treatment the sample was centrifuged at 13000 rpm for 10 min in BiofugeA. Supernatant was taken and the phenol-chloroform (phenol : chloroform : isoamyl alcohocol = 25:24:1) extraction has been carried out until the sample was purified from proteins, carbohydrates and lipids. After the purification RNAse was added (50 μg/ml), incubated at 37°C for 15 min, and the phenol-chloroform extraction was repeated. Nucleic acids were concentrated by 0.3 M Na-acetate pH 5.2 (1/10 of volume) and isopropanol (0.7 of volume). After incubation (-20°C over night or -80°C for 20 min) and centrifugation (13000 rpm for 5 min) the pellet has been washed with ethanol (75% 1 ml, -20°C), centrifugated again (13000 rpm for 5 min), air dried and resuspended in mili Q water.

Isolation of DNA from lactococci and lactobacilli

Total DNA from lactococci and lactobacilli was isolated by modified method described by Hopwood (Hopwood *et al.,* 1985.). Pellet of log culture (OD_{600}=0.6-0.8) was resuspended in 0.5 ml lysozyme solution (6.7% sucrose, 50 mM Tris-HCl,

1 mM EDTA, final pH 8.0). Concentration of lysozyme was 8 mg/ml for lactobacilli and 4 mg/ml for lactococci, RNAse 50 μg/ml. Cells were incubated at 37^{o}C for 30 min, and then 250 μl of 2% SDS was added (vortex 1 min). Following cell lysis phenol-chloroform extraction was performed until the solution was almost protein free. DNA was concentrated with 0.3 M Na-acetate pH 4.8 (1/10 of volume) and isopropanol (0.7 of volume). After incubation (-20^{o}C over night or -80^{o}C for 20 min) and centrifugation (13000 rpm for 5 min) the pellet has been washed with ethanol (75% 1 ml, -20^{o}C), centrifuged again (13000 rpm for 5 min), air dried and resuspended in mili Q water.

Isolation of plasmid DNA from E. coli

Plasmid DNA was isolated using JETSTAR Plasmid Kit developed by GENOMED.

PCR amplification procedure

Set of primers used in this study were designed from the 16S rRNA gene sequences available from GeneBank. Primers were chosen to be complementary to the variable region of this gene and were named P116S (5'-GGAATCTTCCACAATGGACG-3') and P216S (5'-TGACGGGCGGTGTGTACAAG-3'). PCR amplification was performed with the *Taq* DNA polymerase kit from Amersham Pharmacia Biotech. PCR reaction was set up containing: 0.1-1 μg of total DNA isolated from cheese in 1x reaction buffer (RB: 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100 finaly pH 9.0 at 25^{o}C), dNTP mixture (200 μM each), 0.25 μM each primer, and 1U of DNA *Taq* polymerase. The amplification conditions were: first denaturation at 94^{o}C for 5 min and then 30 cycles of three steps – denaturation at 94^{o}C for 1 min, annealing at 45^{o}C for 1 min, polymerisation at 72^{o}C for 1 min, and finally polymerisation at 72^{o}C for 10 min.

Cloning of the PCR fragments from total DNA isolated from cheese

Clone libraries of the 16S rDNA amplicons from cheese were constructed. Amplicons derived from PCR of 16S rDNA were purified using PCR product purification kit, and then were treated with Klenow Fragment and T4 Polynucleotide Kinase. The whole procedure, including ligation of fragment into *Sma*I digested and dephosphorilated pUC18, was performed by SureClone Ligation Kit (Amersham Pharmacia Biotech).

RFLP (Restriction fragment length polymorphism)

PCR fragments amplified from DNA of *Lactococcus lactis* MG1363*, Lactobacillus paracasei* BGHN14 and *Lactobacillus helveticus* CNRZ32 and amplicons from total DNA isolated from cheese were compared using RFLP. Enzymes used for comparison were: *Alu*I, *Eco*RI, *Hae*III i *Msp*I. For all enzymes, except *Eco*RI, digestions were performed in 1xM buffer , and for *Eco*RI digestion we used 1xH buffer (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

Lactic acid bacteria (LAB) are of eminent economic importance because of their widespread use in food and feed fermentations. Isolation of new LAB strains or species, and the discovery those of industrial importance is the reason that determination of LAB was our primary target. Testing for the presence of strains in their natural habitats is one of the ways to attain the goal (Zoetendal *et al.,* 1998). The most critical step of the whole procedure is the extraction of total DNA, since all further analysis assume the complete and representative presence of accessible nucleic acids (Vaughan *et al.,* 2000). We developed a succesfull method for the isolation of total DNA from cheese, to investigate the diversity of the dominating microbial communities of Doboj semi-hard homemade cheese. The method used in this study was a bead beating one, with modifications due to high fat content of this cheese (Randazzo *et al*. 2002). Successive removal of fat layer with sterile glass rods with cotton wool tips was the modification of the original method. The quantity and quality of DNA (Figure 1.) extracted by this method was sufficient for further experiments. This method, with extra modifications, could also be used for extraction of RNA from cheese samples, this will be our future work: extraction of RNA and RT-PCR. PCR amplification, with P116S and P216S primers for 16S rRNA genes from lactobacilli, performed in a way that the specificity of annealing reaction was reduced to 45°C to allow amplification of the lactococcal sequences, as well. Equal amplification efficiency of 16S rDNA was necessary to get an exact insight into the microbial composition of the cheese, but it has been reported, when mixtures of DNA were used as templates, it resulted in bias amplification. In due to avoid unequal amplification we decreased the number of amplification cycles and used high template concentration. DNA from cheese microflora, and DNA from referent strains (*Lactococcus lactis* MG1363*, Lactobacillus paracasei* BGHN14 and *Lactobacillus helveticus* CNRZ32) were used as a template in PCR reaction. The PCR products of 1040 bp in length were obtained (Figure 2.). After extraction and purification from the gel, PCR products were blunted and treated with T4 Polynucleotide Kinase and ligated into the pUC18 vector digested with *Sma*I. Competent cells of *E. coli* DH5α strain were transformed with ligation mixtures and transformants were selected on LB plates containing ampicillin (100 μg/ml) and X-gal (0.002%). White (n=49) and light-blue (n=40) transformants were obtained and used for further analysis. Plasmid DNA from white and light blue transformants was isolated and digested with *Eco*RI and *Hin*dIII restriction enzymes. Restriction analysis of plasmid DNA from light-blue transformants revealed there was no ligated PCR

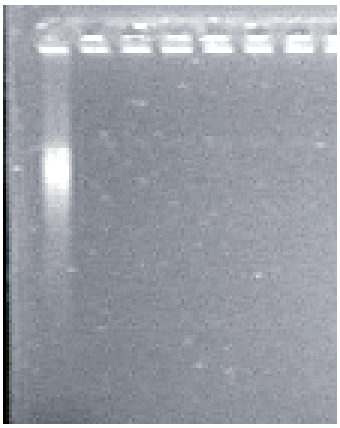

Figure 1. Electrophoresis of total DNA isolated directly from cheese sample (1% agarose)

fragment in it (data not shown). ontrary, analysis of plasmid DNA from white transformants revealed that the most of analysed clones contained fragments of 700 bp and 350 bp (Figure 3.), indicating the presence of *Eco*RI restriction site. Only one of 49 analysed clones (from white transformants) contained DNA fragment of 1040 bp (pBJ42, Figure 4.), and was further analysed by RFLP with restriction enzymes *Alu*I, *Eco*RI, *Hae*III i *Msp*I. Amplicons from DNA of referent strains (*Lactococcus lactis* MG1363*, Lactobacillus paracasei* BGHN14 and *Lactobacillus helveticus* CNRZ32) were used for comparison with pBJ42. Results of RFLP analysis revealed that when the PCR product obtained with *L. lactis* MG1363 DNA was digested with *Eco*RI restriction enzyme it gave fragments of 700 bp and 350 bp, what indicated that 48 of analysed clones (Figures 3. and 4.) are lactococcal. Digestion with restriction enzyme *Msp*I revealed that DNA fragment from pBJ42 and PCR fragment from *L. lactis* MG1363 had the same *Msp*I restriction profile which indicates that this fragment is also lactococcal, but specific compared to all other lactococcal clones. Digestions with restriction

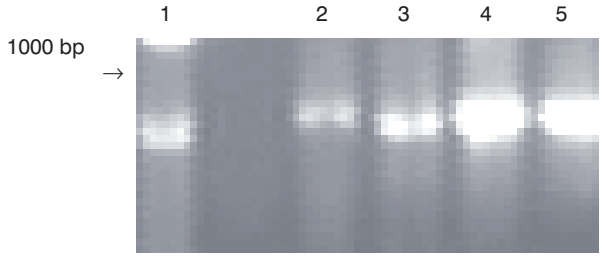

Figure 2. PCR fragments amplified with primers P116S and P216S (on 1% agarose gel):
1. ladder 100 bp;
2. PCR fragment from *L. lactis* MG1363;
3. PCR fragment from *Lb. paracasei* BGHN14;
4. PCR fragment from *Lb. helveticus* CNRZ32;
5. PCR fragment from total DNA from cheese.

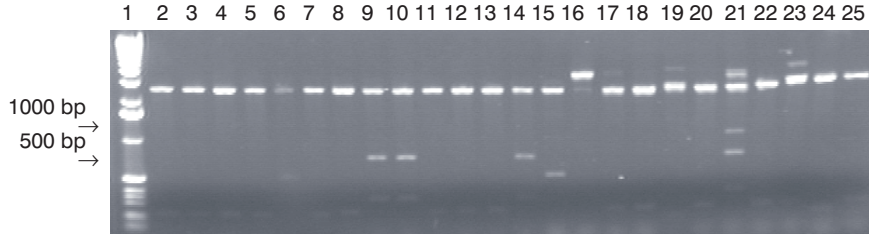

Figure 3. Results of *Eco*RI-*Hin*dIII digestion of plasmid DNA of analysed transformants:
1. ladder 100 bp.; 2-25. analysed clones

enzymes *Alu*I and *Hae*III of PCR fragments were not informative enough (Figure 5.) Future analysis of 16S RNA gene polymorphism should be based on sequencing of clones of interest (especially pBJ42) to see the differences between them at nucleotide level what will reveal their subspecies phylogenetic level.

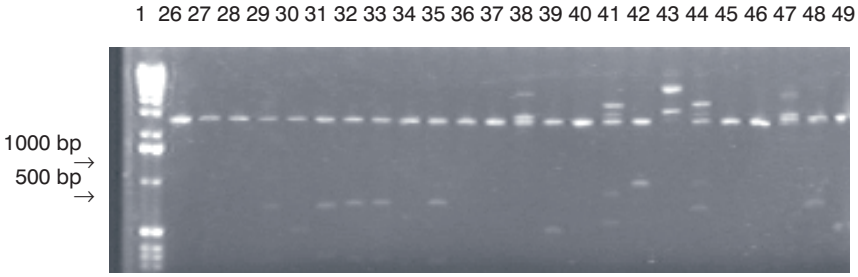

Figure 4. Results of *Eco*RI-*Hin*dIII digestion of plasmid DNA of analysed transformants: 1. ladder 100 bp.; 26-49. analysed clones; 42. pBJ42

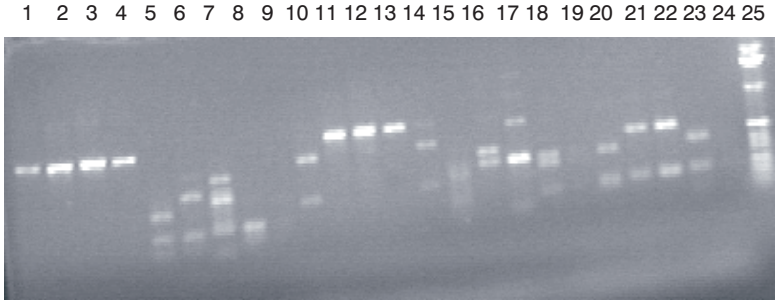

Figure 5. RFLP analysis of PCR products:
-*L. lactis* MG1363 (1- native PCR fragment; 5- *Alu*I digestion of PCR fragment; 10- *Eco*RI digestion of PCR fragment; 15- *Hae*III digestion of PCR fragment; 20- *Msp*I digestion of PCR fragment);
-*Lb. paracasei* BGHN14 (2- native PCR fragment; 6- *Alu*I digestion of PCR fragment; 11- *Eco*RI digestion of PCR fragment ; 16- *Hae*III digestion of PCR fragment; 21- *Msp*I digestion of PCR fragment);
-*Lb. helveticus* CNRZ32 (3- native PCR fragment; 7- *Alu*I digestion of PCR fragment; 12- *Eco*RI digestion of PCR fragment; 17- *Hae*III digestion of PCR fragment; 22- *Msp*I digestion of PCR fragment);
-clone 42 (8 - *Alu*I digestion of PCR fragment; 13 - *Eco*RI digestion of PCR fragment; 18 - *Hae*III digestion of PCR fragment; 23 - *Msp*I digestion of PCR fragment);
-PCR fragment amplified from total DNA from cheese (4 – native PCR fragment; 9- *Alu*I digestion of PCR fragment; 14- *Eco*RI digestion of PCR fragment; 19- *Hae*III digestion of PCR fragment; 24- *Msp*I digestion of PCR fragment);
-25- ladder 100 bp.

In this study, results revealed the dominant presence of the mesophilic *Lactococcus* species. It is well known that some *Lactococcus* strains are highly adapted to the milk enviroment. These strains possess the highly efficient lactose metabolism or proteolytic traits (Kok *et al*., 1985; Vos *et al*., 1989). Therefore, the *Lactococcus* species could be considered as both the dominant and metabolically most important bacteria in this cheese. Furthermore, it appeared that these strains are also involved in the flavour and aroma development of the cheese and are well adapted to the particular environmental conditions of cheese ripening process. All these findings make the *Lactococcus* species present in this cheese good starters in the dairy industry.

Address for correspondence:
Jovčić Branko, MSci
Institute of Molecular Genetics and Genetic Engineering,
Vojvode Stepe 444a
P.O. BOX 23,
11010 Belgrade,
Serbia & Montenegro
e-mail: lab6@eunet.yu

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MOLEKULARNA KARAKTERIZACIJA SIRA PRIPREMLJENOG U DOMAĆINSTVU

JOVČIĆ B, GOLIĆ NATAŠA, KOJIĆ M i TOPISIROVIĆ LJ

SADRŽAJ

U poslednjoj deceniji zabeležen je nagli razvoj molekularnih tehnika baziranih na 16S i 23S rRNK, koje se koriste u izučavanju biodiverziteta mikroorganizama. U ovom radu ispitivana je mikroflora polutvrdog sira pripremljenog u domaćinstvu. Zbog visokog sadržaja masti u ovom siru razvili smo novu tehniku za izolaciju totalne DNK iz sira (metod je baziran na bead beating-u). Brza izolacija DNK iz mikroflore sira omogućila nam je molekularnu identifikaciju BMK (BAKTERIJE MLEČNE KISELINE) na osnovu umnožavanja gena za 16S rRNK PCR metodom. Za umnožavanje su korišćeni prajmeri specifični za gene 16S rRNK laktobacila, ali su uslovi PCR reakcije bili takvi da su omogućavali i umnožavanje gena 16S rRNK laktokoka. Rezultati RFLP analize pokazali su da mikrofloru sira pripremljenog u domaćinstvu čine predominantno laktokoke.