Journal of Dairy Research (2012) **79** 26–32. © *Proprietors of Journal of Dairy Research 2011*
doi:10.1017/S0022029911000677

Cascade cell lyses and DNA extraction for identification of genes and microorganisms in kefir grains

Magdalena Kowalczyk[1]*†, Piotr Kolakowski[2]†, Joanna M Radziwill-Bienkowska[1], Agnieszka Szmytkowska[3] and Jacek Bardowski[1]

[1] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland
[2] Danisco Biolacta, Innovation, Olsztyn, Poland
[3] Department of Pharmaceutical Microbiology, Medical University of Warsaw, Warsaw, Poland

Received 7 March 2011; accepted for publication 20 August 2011; first published online 13 October 2011

Kefir is a dairy product popular in many countries in Central Europe, especially in Poland and other countries of Eastern and Northern Europe. This type of fermented milk is produced by a complex population of symbiotic bacteria and yeasts. In this work, conditions for DNA extraction, involving disruption of kefir grains and a cascade of cell lysis treatments, were established. Extraction procedure of total microbial DNA was carried out directly from fresh kefir grains. Using different lysis stringency conditions, five DNA pools were obtained. Genetic diversity of DNA pools were validated by RAPD analysis, which showed differences in patterns of amplified DNA fragments, indicating diverse microbial composition of all the analysed samples. These DNA pools were used for construction of genomic DNA libraries for sequencing. As much as 50% of the analysed nucleotide sequences showed homology to sequences from bacteria belonging to the *Lactobacillus* genus. Several sequences were similar to sequences from bacteria representing *Lactococcus, Oenococcus, Pediococcus, Streptococcus* and *Leuconostoc* species. Among homologues of yeast proteins were those from *Candida albicans, Candida glabrata, Kluyveromyces lactis* and *Saccharomyces cerevisiae*. In addition, several sequences were found to be homologous to sequences from bacteriophages.

Keywords: Complex ecosystems, kefir grains, cascade cell lysis, DNA isolation, biodiversity.

Kefir is a fermented milk food product that originated in the Caucasus. Due to its unique aroma and flavour, nutritional value and potential probiotic properties, it is becoming increasingly more popular all over the world. Kefir is produced with the use of kefir grains, representing a specific combination of various bacteria and yeasts. The microbial composition of kefir grains has not been fully elucidated and attempts to produce synthetic kefir grains from isolated microorganisms have so far been unsuccessful (Garrote et al. 2010). It is worth emphasising that the microbial content of kefir grains depends on their origin, storage conditions and handling pattern (Pintado et al. 1996).

Most studies on biodiversity of kefir or kefir grains were based on classical microbiological methods using selective growth media. The resultant identification of microbes could have been inadequate because certain organisms are uncultivable. Additionally, in complex microbial ecosystems such as kefir grains, symbiotic interactions between yeasts and lactic acid bacteria have been observed (Lopitz-Otsoa et al. 2006). Thus, molecular techniques based on culture independent methods offer new possibilities for determining the microbiological composition of kefir grains. Recent publications identifying the microorganisms in kefir grains by culture independent methods demonstrated the results of DGGE analysis of bacterial 16S rDNA amplicons or yeast 26S rDNA generated directly from kefir grains. Garbers et al. (2004) identified, using specific primers and total DNA isolated with glass beads as template, several species belonging to the *Lactobacillus* genus and two yeasts from *Candida* and *Saccharomyces*. In spite of limitations of the DGGE analysis, they identified two uncultivable strains and two species previously not detected in kefir grains (Garbers et al. 2004). Other bacteria were identified by a Chinese group (Wang et al. 2006); nucleotide sequences of DNA bands generated by DGGE were highly homologous to those of bacteria from the genus of *Sphingobacterium, Lactobacillus, Enterobacter* and *Acinetobacter*. In another study, total DNA of bacterial strains from three different kefir grains were extracted using a blood and tissue genomic extraction system and directly identified by PCR-DGGE

*For correspondence; e-mail: mk@ibb.waw.pl
†The first two authors contributed equally to this article.

(Chen et al. 2008). The results indicated the presence of *Lb. kefiranofaciens* in all analysed samples, *Lb. kefiri* in one, and *Lactococcus lactis* in two. Culture-independent method revealed also two non LAB species in these kefir grains not detected by culturing: *Escherichia coli* and *Pseudomonas* spp. However, the varieties of the LAB strains identified by PCR-DGGE culture-independent method were fewer than those identified with the use of initial enrichment stage on nutritive media (Chen et al. 2008). Zhou et al. (2009) analysed the microflora in Tibetan kefir grains using the FTA® membrane-based method combined with PCR-DGGE. They showed that the dominant microorganisms of these kefir grains were *Pseudomonas* spp., *Leuconostoc mesenteroides, Lb. helveticus, Lb. kefiranofaciens, Lb. kefiri, Lb. casei, Lc. lactis, Kasachstania unispora, Kluyveromyces marxianus, Sac. cerevisiae*, and *Kazachstania exigua*. Previously, the membrane card-based method was evaluated for different types of raw milk and dairy food products, with specific primers for dominant bacterial strains or species (Tilsala-Timisjarvi & Alatossava, 2004). The comparison of the FTA® protocol with conventional DNA isolation showed that both methods were very similar except in the case where the *Lb. rhamnosus* GG was detected in yoghurt with strain-specific primers only by the FTA® method. This method combined with PCR-DGGE with general bacterial or yeast primers applied for kefir grains by Zhou et al. (2009) allowed the identification of several dominant microorganisms. However, the completeness of this analysis is difficult to assess as data enabling the comparison with culture dependent or other methods were not presented. Thus, taking into account the big diversity of different kefir grains and the limited number of molecular analyses, culture independent methods ought to be used for further comprehensive studies of microbial composition of kefir grains. One of culture-independent methods that enables a comprehensive view of the genetic diversity is metagenomics. In this approach DNA is isolated directly from environmental samples and sequenced. High-throughput sequencing methods make shotgun sequencing of the whole metagenome from environmental samples informative. Shotgun sequencing of DNA isolated from complex environments can be used for identification of microorganisms, pool of genes and for prediction of proteins from global sampling data (Hutchinson, 2007). The metagenomic approach has been applied to study microbial populations in many environments including an acid-mine biofilm (Tyson et al. 2004), Sargasso Sea (Venter et al. 2004) or the human gut (Gill et al. 2006). To our knowledge, there are no published data pertinent to shotgun sequencing of genomic libraries for studying Kefir grains.

The aim of this study was to optimise the DNA extraction procedure for efficient identification of gene and microbial biodiversity in the complex microbial consortium present in kefir grains. Our goal was to isolate DNA from fresh material. The proposed culture-independent approach is based on microbial enrichment at the level of DNA extraction. The procedure comprises a cascade of several cell lyses in order to isolate DNA from diverse and also less prevalent organisms. Such DNA pools were used for preparation of genomic DNA banks for sequencing and could also be used for sequencing of 16S or 26S rRNA-coding DNA fragments, which should increase the chance of identification of various microorganisms, not only those which dominate this ecosystem.

Materials and Methods

Culturing of kefir grains

Samples of kefir grains supplied by Danisco Biolacta, Olsztyn, Poland were cultured in 10% skimmed milk, pH 6·6, and sterilised for 30 min at 95 °C, according to the method described by Oklinski (2006). Briefly, 600 ml of the milk medium was inoculated with 60 g of fresh kefir grains. After 22 h incubation at 18 °C kefir grains were drained in a sieve, washed with sterile water (3 vols water to 1 vol culture medium) and drained on sterile paper towel for 10 min. Subsequently, kefir grains were weighed and a portion of 60 g was used for inoculation of a new batch of medium. A series of passages were performed until kefir grains attained appropriate characteristics, including medium pH (4·0–4·3) and increase in biomass (12–18%), pointing to full activity of kefir grains.

Homogenisation of kefir grains

Sterile 0·9% NaCl solution was added to 20 g washed and drained active kefir grains to the final volume of 200 ml and homogenised at 24 000 rpm for 5 min in laboratory homogeniser H500 (POL-EKO-APARATURA, Poland).

Isolation of DNA

Homogenised kefir grains were subjected to a gradual change in cell lysis conditions – a cascade of cell lysis. A 2 ml sample of homogenised kefir grains was harvested for 1 min at 8944 ***g*** in the microcentrifuge. The pellet was resuspended in 300 µl TES buffer (25 mM Tris; 10 mM EDTA; 50 mM sucrose) and 150 µl 20% SDS – lysis A. After 5 min incubation at room temperature the sample was centrifuged. The supernatant was used for DNA extraction and the cell pellet was subjected to lysis step B (Fig. 1). Lysis step B was based on the method of DNA isolation from *Lc. lactis* (Tailliez et al. 1998). The pellet after lysis A was resuspended in 300 µl TES solution containing 20 mg lysozyme/ml (cat. no. 62971, Fluka, Belgium) and incubated for 30 min at 37 °C. Then, 150 µl 20% SDS was added, the total was incubated for 5 min at room temperature and centrifuged. Lysis C was performed similarly to lysis B, except that 15 µl of 1 U mutanolysin/µl (cat. no. M9901, Sigma-Aldrich Co., USA) was added to the TES-lysozyme solution and incubation was prolonged to 1 h. This method has been used for DNA isolation from several *Lactobacillus* spp. (Fliss et al.

https://doi.org/10.1017/S0022029911000677 Published online by Cambridge University Press

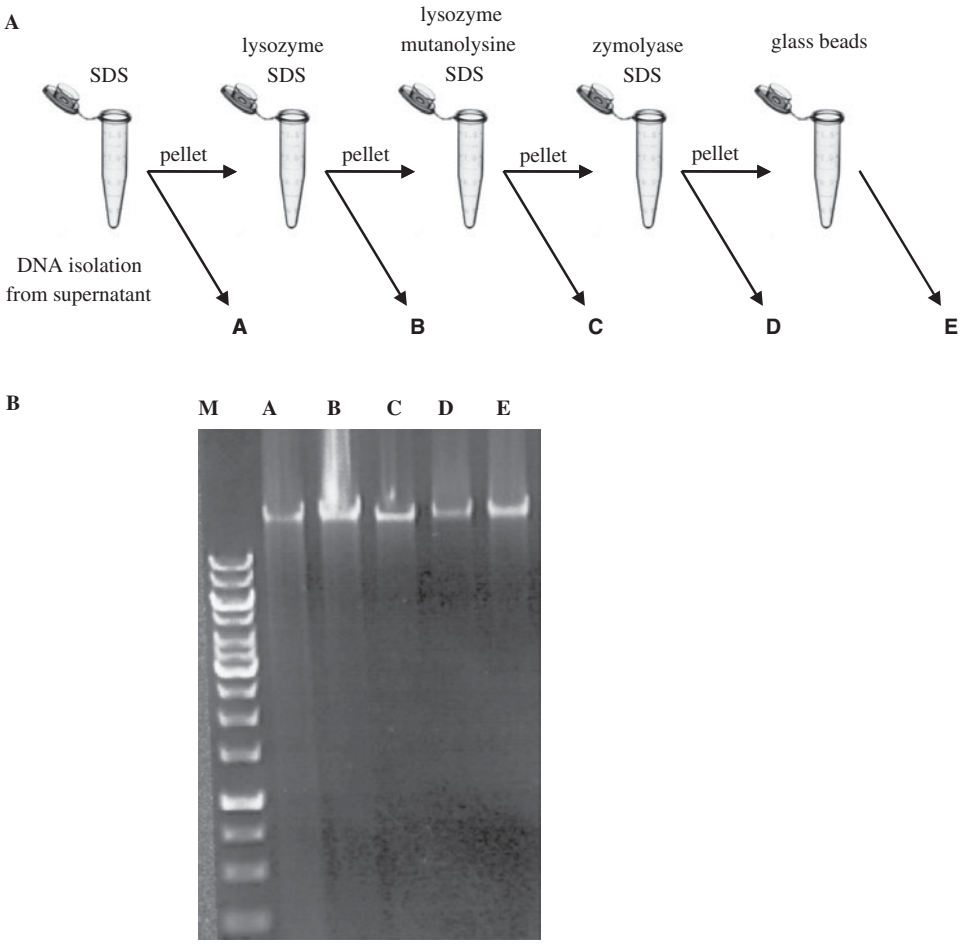

Fig. 1. Cascade cell lysis for DNA extraction (pools A, B, C, D, E) from kefir grains. (A) Gradual change in cell lysis conditions; (B) electrophoresis of DNA in 0·8% agarose gel visualised under UV light. Chromosomal DNA pools (A, B, C, D, E), M: GeneRuler 1 kb DNA Ladder (Fermentas).

1991). Lysis D was based on the method of DNA isolation from *Sac. cerevisiae* (Sherman et al. 1986). The pellet after lysis C was resuspended in 0·5 ml SE buffer (1 M sorbitol; 0·1 M EDTA; pH 7·5) and 20 µl of 5 mg zymolyase/ml (cat. no. 320932, ICN, Costa Mesa, CA, USA) was added – lysis D. After 1 h incubation at 37 °C the sample was centrifuged. The pellet was resuspended in 0·5 ml TE buffer (50 mM Tris; 20 mM EDTA; pH 7·4) with 50 µl 10% SDS, incubated for 30 min at 65 °C and centrifuged at 8944 ***g*** for 1 min. Finally, the pellet after lysis D was resuspended in 400 µl TES buffer and the cells were mechanically disrupted with glass beads (100 µm, 0·6 g) by three 1-min pulses in MBB-8 apparatus (Biospec, USA) with 1 min cooling intervals – lysis E. To inhibit DNA degradation, 1 µl diethylpyrocarbonate (DEPC) was added for every 100 µl of the sample just before addition of SDS (in lyses A, B, C and D) and before mechanical disruption of cells (for lysis E). In addition, 1 µl of 20 mg proteinase K/ml (cat. no. 124568·0100, Merck, Germany) was added for every 100 µl of supernatant from every lysis step, and samples were then incubated for 30 min at 37 °C. DNA in supernatants from lyses A, B, C and E was purified by phenol/chloroform extraction (Sambrook et al. 1989), whereas the supernatant from lysis D was incubated for 15 min on ice with 200 µl 5 M potassium acetate and centrifuged for 15 min at 17 530 ***g*** at 4 °C in a microcentrifuge.

Finally, 1 ml isopropanol was added to the water phase after phenol/chloroform extraction and to the supernatant of the centrifuged sample D. DNA was precipitated over-night at – 20 °C. After centrifugation for 30 min at 17 530 ***g*** at 4 °C, the pellet was washed with 70% ethanol, dried, dissolved in 60 µl RNase (100 µg/ml) for lyses A, B, C or in 75 µl for lyses D, E and incubated 15 min at 37 °C. The extracted DNA was stored at – 20 °C until required.

DNA electrophoresis

DNA was analysed by electrophoresis in TAE buffer on 0·8–1·0% agarose gel supplemented with 0·1 µg ethidium bromide/ml (Sambrook et al. 1989). After electrophoresis, gels were photographed under UV light for documentation.

https://doi.org/10.1017/S0022029911000677 Published online by Cambridge University Press

RAPD fingerprinting

RAPD-PCR was carried out in a final volume of 30 µl reaction mixture containing: 20 pmol of each primer, 250 µM of each of the deoxynucleotide triphosphates (TaKaRa Bio Inc., Japan), 2 U of TaKaRa ExTaq polymerase (TaKaRa Bio Inc.) and 0·5 µg of template DNA (from each of A–E DNA pools) in 1 × PCR buffer. Two decamer primers, RAPD-B06 (Tailliez et al. 1998) or RAPD-M13 (Andrighetto et al. 2000; Rossetti & Giraffa, 2005), were used to generate random amplicons. The PCR consisted of an initial denaturation step at 94 °C for 5 min, followed by 30 cycles comprising heat denaturation at 94 °C for 1 min, primer annealing at 42 °C for 2 min and extension at 72 °C for 2 min; it was completed with a final extension step at 72 °C for 7 min, after which samples were held at 4 °C in a PTC 200 Thermal Cycler (MJ Research, USA).

Preparation and validation of DNA banks

The extracted DNA was sheared and subcloned using the TOPO Shotgun Subcloning Kit according to the manufacturer's instructions (Invitrogen Corporation). 2 µg of each DNA pool (A, B, C, D or E) was sheared in 750 µl Shearing Buffer (TE, pH 8 containing 20% glycerol) in nebulisers connected to compressed helium. Undiluted, blunt-end, dephosphorylated DNA was cloned into pCR 4Blunt-TOPO. The TOPO cloning reactions were dialysed 15 min on MF-Millipore Membrane Filters 0·025 µm (Millipore Corporation) and transformed into One Shot TOP10 Electrocomp *Escherichia coli* (Invitrogen Corporation). Transformed bacteria were plated on selective LB medium (60 µg/ml X-Gal; 0·3 mM IPTG; 50 µg/ml ampicillin) and incubated overnight at 37 °C. Then, 30 white colonies from each DNA bank were analysed for the presence of inserts with a colony PCR technique. Colonies were picked and suspended in 20 µl reaction mixture in 1 × PCR buffer containing: 10 pmol of each of the two universal primers – M13/pUC19, 250 µM of each of the deoxynucleotide triphosphates (Polgen, Poland) and 1 U of Taq polymerase (Polgen). Reaction mixtures were first incubated at 94 °C for 10 min to lyse the cells and inactivate nucleases. Then, amplifications were done for 30 cycles, with each cycle consisting of 94 °C step for 1 min, 50 °C step for 1 min and 72 °C step for 2 min. For the final extension, samples were incubated at 72 °C for 10 min and held at 4 °C.

After confirming the quality of the DNA banks, bacteria growing as white colonies were frozen in 100 µl LB medium with 10% glycerol and 100 µg ampicillin/ml, in 96-well microtiter plates. Five DNA banks (A, B, C, D, E), each in duplicate, were kept at − 80 °C for later analysis.

Sequencing analysis

Sequencing of DNA inserts of 96 clones from each DNA bank (A, B, C, D and E) was done by the Sanger method (Sanger et al. 1977). The sequencing products were analysed by the ABI 3730/xl Genetic Analyzer in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences.

For bioinformatic analyses the following programs were used: Clone Manager 9 Professional Edition, PhredPhrap/Consed (Genome Science Department, University of Washington, USA) and BLAST (NCBI).

Results

Processing of kefir grains for DNA extraction

The first attempt to isolate DNA from kefir grains was undertaken using homogenised kefir grains stored at 4 °C in 0·9% NaCl ('stored grains'). However, stored grains generated problems during the lysis procedure in terms of their viscosity and with separation of water and phenol/chloroform phases due to the presence of exopolysaccharides. This resulted in a very low yield and poor quality of obtained DNA. However, when fresh kefir grains were used, both DNA quality and yield were much better than in the case of stored grains. Thus, DNA was isolated from fresh and active kefir grains cultured and homogenised directly before extraction, as described in Materials and Methods.

Efficiency of the cascade cell lysis procedure

The applied DNA extraction method relied on a gradual change in cell lysis conditions, termed here as cascade cell lysis and described in detail in Materials and Methods. The efficiency of the cascade cell lysis was monitored by microscopic observations of cell pellets obtained after each lysis step. Microbial biodiversity of the cell pellet was examined by assessing the cell number, morphology (rod, coccus, yeast cells) and form (single, chain, aggregate) observed under a phase-contrast microscope. In homogenised kefir grains, cocci and rods forming various types of structures (chains, aggregates) as well as much larger yeast cells were observed. Biodiversity of microorganisms was observed to decrease during the applied cell lysis procedure. At the end of lysis D, rod shaped bacteria were the only morphological forms visible in the pellet. Due to large amounts of cell pellet forming after centrifugation of homogenised kefir grains, it was necessary to increase the volume of reagents used. In addition, in order to decrease DNA degradation, DEPC – a nuclease inhibitor, and proteinase K were added. In effect, five DNA pools, A–E, were obtained (Fig. 1B). The quality of DNA preparations was examined by agarose gel electrophoresis. DNA bands visible under UV corresponded well to the position of the chromosomal DNA in the gel and confirmed the good quality of the isolated DNA. The amount of DNA was measured with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). The following concentrations were obtained: A = 1·6 µg/µl, B = 0·5 µg/µl,

https://doi.org/10.1017/S0022029911000677 Published online by Cambridge University Press

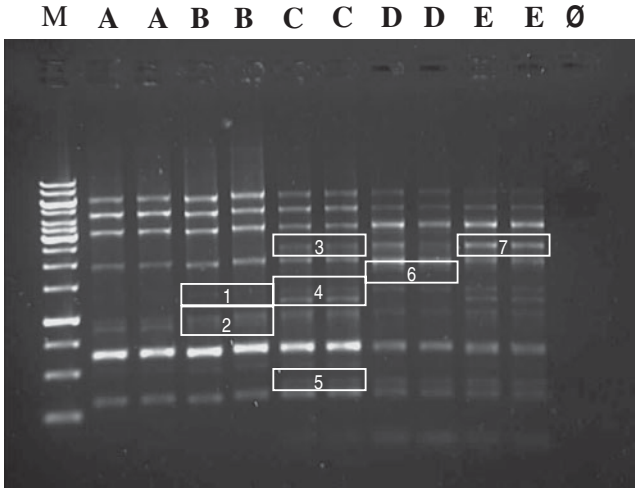

Fig. 2. RAPD-PCR electrophoretic patterns in 1% agarose gels obtained for different DNA pools (A, B, C, D, E) with primer B06 (5′-TGCTCTGCCC-3′). Ø, negative control for PCR; M, GeneRuler 1 kb DNA Ladder (Fermentas). The most important differences between the following pools were marked with white frames; B:A (frames 1 + 2), C:B (frames 3 + 4 + 5), D:C (frame 6), E:D (frame 7).

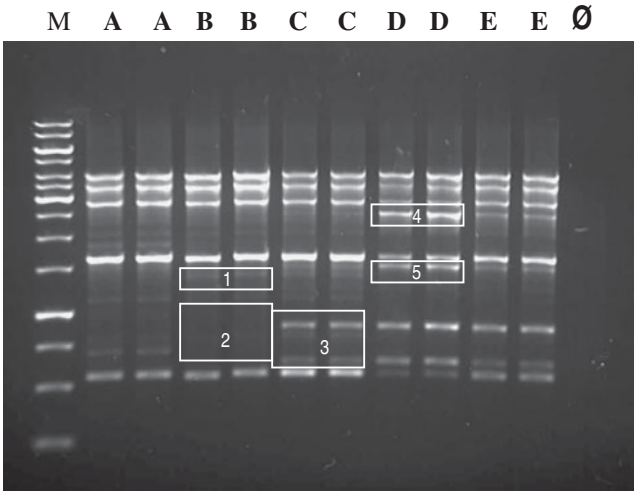

Fig. 3. RAPD-PCR electrophoretic patterns in 1% agarose gels obtained for different DNA pools (A, B, C, D, E) with primer M13 (5′-GAGGGTGGCGGTTCT-3′). Ø, negative control for PCR; M, GeneRuler 1 kb DNA Ladder (Fermentas). The most important differences between the following pools were marked with white frames; B:A (frames 1 + 2), C:B (frame 3), D:C (frames 4 + 5).

C = 0·2 μg/μl, D = 0·3 μg/μl, E = 0·3 μg/μl, and they were found to be sufficient for preparation of DNA banks.

Validation of DNA pools by RAPD analysis

To validate that the different DNA pools obtained represent different sets of microorganisms, RAPD-PCR analyses with primers RAPD-B06 (Fig. 2) or RAPD-M13 (Fig. 3) and five DNA pools as templates were performed. The RAPD-B06 primer has been commonly used for classification of *Lc. lactis* strains (Tailliez et al. 1998), and the RAPD-M13 primer has been used for classification of different microorganisms, including yeasts (Andrighetto et al. 2000; Rossetti & Giraffa, 2005).

Differences in DNA band patterns generated by RAPD-PCR and in the intensity among DNA pools were clearly visible (Figs. 2 & 3). The most important differences between DNA pools were marked with white frames. Analysis of DNA patterns generated with primer B06 showed that several DNA bands were present in all five DNA pools, while some of them were highly represented in A, B and C pools, but not in the last two pools (Fig. 2). The higher intensity of bands obtained in pools A, B and C compared with the same obtained in pools D and E could be due to the higher amount of DNA template corresponding to particular microorganisms, which indicates their preferential extraction in the first pools. Interestingly, there were also bands characteristic for particular DNA pools. For pool B, two differences were observed compared with pool A. The first difference, marked by the white frame 1, concerns a barely visible band that appeared in pool B; the second difference, in frame 2, concerns the reversed intensity of two bands. The lower band in this frame was no longer visible for DNA pools C-E. Moreover, two novel bands appeared in pool C which were absent in pool B (frames 3 and 5). In addition, the intensity of the lower band, corresponding to one of the bands observed in frame 1, was much higher (frame 4). A similar DNA electrophoretic pattern to that generated for pool C was observed for pool E; yet, it differed in the intensity of one band (frame 7). Unique DNA bands were also observed in the case of the second primer – RAPD-M13 (Fig. 3). A novel previously unobserved DNA band appeared in pool B compared with pool A (frame 1). However, at the same time several bands observed for pool A were no longer visible in pool B (frame 2) as well as in other DNA pools. Besides that three DNA bands appeared in pool C (frame 3), where the middle band seems to be unique only for this DNA pool. Finally, two other differences were observed between pools D and C (frames 4 and 5), with much higher intensity of the two DNA bands in pool D.

Overall, the RAPD fingerprinting method allowed discrimination between the five DNA pools isolated from kefir grains.

Implementation of the cascade cell lysis for semi-massive sequencing of genomic DNA

DNA pools were used to prepare representative DNA banks (A, B, C, D and E) using the TOPO Shotgun Subcloning Kit according to the manufacturer's instructions (Invitrogen Corporation). Nebulisations resulted in generation of DNA fragments ranging from 0·5 to 8 kb. The quality of the DNA banks was tested by colony PCR for thirty transformants of each bank. The amplified fragment of the vector without insert was expected to have 212 bp. For all the DNA banks

https://doi.org/10.1017/S0022029911000677 Published online by Cambridge University Press

Table 1. Number of nucleotide sequences homologous to amino acid sequences from different groups of microorganisms. Results for the identity of more than 50% and more than 70% (in brackets)

	Lactobacillus spp.	Other bacteria	Yeasts	Bacteriophages	Total/No. of all sequences
Bank A	55 (42)	2 (0)	1 (1)	4 (1)	62 (44)/95
Bank B	43 (37)	5 (2)	0 (0)	4 (0)	52 (39)/89
Bank C	49 (45)	2 (2)	4 (1)	1 (0)	56 (47)/88
Bank D	27 (25)	1 (0)	14 (3)	0 (0)	42 (28)/78
Bank E	41 (36)	0 (0)	5 (2)	0 (0)	46 (38)/77

most of the amplified inserts were longer than 500 bp, with the longest fragment cloned having 3 kb.

Ninety six clones from each DNA bank were sequenced, and bioinformatic analyses of the obtained sequences, including removal of fragments of vector sequences, were done using the Phred/Phrap/Consed package. The 427 sequences obtained were compared with those available in NCBI using the BLAST algorithm version blastx (Altschul et al. 1997). Each blast alignment was filtered for three hits with the highest score value, with sequence identity above 50 or 70% (at least 30 amino acids identical) and *E*-value thresholds of 10^{-3}. As much as 50% of the analysed nucleotide sequences, 215 of 427, showed homology to sequences from bacteria belonging to the *Lactobacillus* genus (Table 1). Several sequences were similar to sequences from bacteria representing *Lactococcus, Oenococcus, Pediococcus, Streptococcus* and *Leuconostoc* species (data not presented). Among homologues (above 70% of sequence identity) of yeast proteins were those from *Cand. albicans, Cand. glabrata, K. lactis* and *Sac. cerevisiae*. In addition, several sequences were found to be homologous to sequences from bacteriophages. However, 98 nucleotide sequences longer than 500 bp (including 54 sequences longer than 1000 bp) had no identified homologues.

Discussion

Different methods can be used to determine microbial biodiversity. Classical, non-direct methods based on the culturing of microorganisms on different rich and selective media have to be complemented by direct estimation of microbial biodiversity in the sample. Direct approaches are particularly important for complex populations like kefir grains. In such ecosystems, there are uncultivable organisms requiring symbiotic interactions, which are believed to be difficult to reconstruct.

Total DNA isolated from kefir grains contains large amounts of DNA from different microbial groups (bacteria and yeasts) that have the potential to interfere with specific amplification of particular DNA (Chen et al. 2008). Moreover, dominant species may yield greater amounts of template DNA and, therefore, have a higher probability of detection (Prakitchaiwattana et al. 2004). The detection limitations in determining microbial biodiversity by PCR approaches can also be the consequence of the interactions of proteins and aged cultures with genomic DNA, thereby affecting primer annealing to the template or the activity of DNA polymerase (de Barros Lopes et al. 1996; Beh et al. 2006). For these reasons it is very important to pay attention to DNA extraction protocols and to preparation of DNA templates.

In this study we proposed a culture-independent approach based on microbial enrichment that could be applied to particular cases for various types of dairy products. Cascade cell lysis could be useful for identification of species with a small population size in a mixture of other abundant microorganisms.

Elaboration of the method of DNA isolation directly from kefir grains was the first step toward determining the biological diversity of this complex ecosystem. Kefir grains were cultured according to the procedure used by the grains producer, as published by Oklinski (2006). Preservation of original culture conditions and storage of grains were essential in order to increase the probability of obtaining reproducible and reliable results. These parameters influence the number and type of microorganisms isolated from kefir grains (Garrote et al. 1997; Witthuhn et al. 2005a, b). In order to increase the likelihood of isolating DNA from microorganisms which occur in grains in small quantities, DNA was isolated in several pools. This novel methodological approach – cascade cell lysis, was validated by RAPD analysis. Differences in DNA band patterns confirmed the diversified representation of microbial DNA in each DNA pool. These DNA pools were used to create DNA banks for direct sequencing.

It was expected that bank A represents DNA from microorganisms whose cells lysed easily or whose cell walls were partially disrupted during homogenisation. Other DNA banks were anticipated to represent DNA originating from cells of various microorganisms which could be destroyed by such factors as: lysozyme, lysozyme and mutanolysin, zymolyase and finally, mechanical disruption. For all the DNA banks, most sequences were homologous to those from bacteria belonging to the *Lactobacillus* genus. The small representation of other microorganisms can be due to the dominance of *Lactobacillus* spp. in kefir grains. The number of species detected in a sample is strongly affected by the number of sequences analysed (Schloss & Handelsman, 2005). The biggest variability in the microbial content was visible for bank D, where several sequences were homologous to yeast proteins and there were fewer

https://doi.org/10.1017/S0022029911000677 Published online by Cambridge University Press

sequences homologous to bacterial proteins (Table 1). The number of already sequenced bacterial and yeast genomes isolated from kefir grains is fairly low, which could explain the lack of homologous sequences to such microorganisms. Interestingly, a few sequences from the first three banks were homologous to bacteriophage proteins (Table 1), which indicates that kefir grains can be a source of new, potentially interesting bacteriophages. These results may be supported by the recently published work of De Antoni et al. (2010) regarding the first isolation of bacteriophages from kefir grains. Finally, the observation that for 98 nucleotide sequences longer than 500 bp no homologues were found suggests that there might be unique sequences of microorganisms in kefir grains that have not yet been sequenced. The other, less probable explanation, could be that these cloned fragments are long non-coding regions.

Concluding, cascade cell lysis is a useful approach for assessing biodiversity of complex microbial ecosystems. By applying this approach in dairy microbial sample analysis, we isolated different pools of DNA and increased the chance for identification of less prevalent microorganisms.

This work was supported by the grant of the Polish Ministry of Science and Higher Education No. N312074 31/3966. We thank our colleagues from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis at the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences for DNA sequencing. We also appreciate the help of Dr Agnieszka Szczepankowska and Prof. Halina Sierakowska for language improvements of this manuscript.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **5** 3389–3402

Andrighetto C, Psomas E, Tzanetakis N, Suzzi G & Lombardi A 2000 Randomly amplified polymorphic DNA (RAPD) PCR for the identification of yeasts isolated from dairy products. *Letters in Applied Microbiology* **30** 5–9

Beh AL, Fleet GH, Prakitchaiwattana C & Heard GM 2006 Evaluation of molecular methods for the analysis of yeasts in foods and beverages. *Advances in Experimental Medicine and Biology* **571** 69–106

Chen HC, Wang SY & Chen MJ 2008 Microbiological study of lactic acid bacteria in kefir grains by culture-dependent and culture-independent methods. *Food Microbiology* **25** 492–501

De Antoni G, Zago M, Vasek O, Giraffa G, Carminati D, Marcó MB, Reinheimer J & Suárez V 2010 *Lactobacillus plantarum* bacteriophages isolated from Kefir grains: phenotypic and molecular characterization. *Journal of Dairy Research* **77** 7–12

de Barros Lopes M, Soden A, Henschke PA & Langridge P 1996 PCR differentiation of commercial yeast strains using intron splice site primers. *Applied and Environmental Microbiology* **62** 4514–4520

Fliss I, Emond E, Simard RE & Pandian S 1991 A rapid and efficient method of lysis of *Listeria* and other gram-positive bacteria using mutanolysin. *Biotechniques* **11** 453, 456–457

Garbers I-M, Britz TJ & Witthuhn RC 2004 PCR-based denaturing gradient gel electrophoretic typification and identification of the microbial consortium present in kefir grains. *World Journal of Microbiology and Biotechnology* **20** 687–693

Garrote GL, Abraham AG & De Antoni GL 1997 Preservation of kefir grains, a comparative study. *Lebensmittel-Wissenschaft und-Technologie* **30** 77–84

Garrote GL, Abraham AG & De Antoni GL 2010 Microbial interactions in Kefir: a natural probiotic drink. In *Biotechnology of Lactic Acid Bacteria: Novel Applications*, ch 18 (Eds F Mozzi, RR Raya & GM Vignolo). Oxford, UK: Wiley-Blackwell

Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM & Nelson KE 2006 Metagenomic analysis of the human distal gut microbiome. *Science* **312** 1355–1359

Hutchinson CA III 2007 DNA sequencing: bench to bedside and beyond. *Nucleic Acid Research* **35** 6227–6237

Lopitz-Otsoa F, Rementeria A, Elguezabal N & Garaizar J 2006 Kefir: a symbiotic yeasts-bacteria community with alleged healthy capabilities. *Revista Iberoamericana de Micologia* **23** 67–74

Oklinski MK 2006 The influence of the medium composition and selected parameters of production for the activity of kefir grains. Master's Thesis. Olsztyn, Poland: The University of Warmia and Mazury

Pintado ME, Lopes Da Silva JA, Fernandes PB, Malcata FX & Hogg TA 1996 Microbiological and rheological studies on Portuguese kefir grains. *International Journal of Food Science and Technology* **31** 15–26

Prakitchaiwattana CJ, Fleet GH & Heard GM 2004 Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. *FEMS Yeast Research* **4** 865–877

Rossetti L & Giraffa G 2005 Rapid identification of dairy lactic acid bacteria by M13-generated, RAPD-PCR fingerprint databases. *Journal of Microbiological Methods* **63** 135–144

Sambrook J, Fritsch EF & Maniatis T 1989 *Molecular Cloning: a Laboratory Manual*, 2nd edition. Cold Spring Harbor, New York, USA: CSHL Press

Sanger F, Nicklen S & Coulson AR 1977 DNA sequencing with chain terminating inhibitors. In *Proceedings of the National Academy of Sciences of the United States of America* **74** 5463–5467

Schloss PD & Handelsman J 2005 Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Applied and Environmental Microbiology* **71** 1501–1506

Sherman F, Fink GR & Hicks JB 1986 *Laboratory Course Manual for Methods in Yeast Genetics*. Cold Spring Harbor, New York, USA: CSHL Press

Tailliez P, Tremblay J, Ehrlich SD & Chopin A 1998 Molecular diversity and relationship within *Lactococcus lactis*, as revealed by randomly amplified polymorphic DNA (RAPD). *Systematic and Applied Microbiology* **21** 530–538

Tilsala-Timisjarvi A & Alatossava T 2004 Rapid DNA preparation from milk and dairy process samples for the detection of bacteria by PCR. *Food Microbiology* **21** 365–368

Tyson GW, Chapman J, Hugenholtz P, Allen EE, Ram RJ, Richardson PM, Solovyev VV, Rubin EM, Rokhsar DS & Banfield JF 2004 Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* **428** 37–43

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson W, Fonts DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffmon J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers YH & Smith HO 2004 Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304** 66–74

Wang YY, Li HR, Jia SF, Wu ZJ & Guo BH 2006 Analysis of bacterial diversity of kefir grains by denaturing gradient gel electrophoresis and 16S rDNA sequencing. *Wei Sheng Wu Xue Bao* **46** 310–313 [Article in Chinese]

Witthuhn RC, Schoeman T & Britz TJ 2005a Characterisation of the microbial population at different stages of Kefir production and Kefir grain mass cultivation. *International Dairy Journal* **15** 383–389

Witthuhn RC, Schoeman T, Cilliers A & Britz TJ 2005b Impact of preservation and different packaging conditions on the microbial community and activity of Kefir grains. *Food Microbiology* **22** 337–344

Zhou J, Liu X, Jiang H & Dong M 2009 Analysis of the microflora in Tibetan kefir grains using denaturing gradient gel electrophoresis. *Food Microbiology* **26** 770–775